OPTIMISING METHODS FOR EMBRYONIC AXIS FIXATION AND MICROPROPAGATION OF *Syzygium cordatum* HOCHST.

by

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ABSTRACT

OPTIMISING METHODS FOR EMBRYONIC AXIS FIXATION AND MICROPROPAGATION OF *Syzygium cordatum* HOCHST.

Syzygium cordatum Hochst. (family - Myrtaceae), commonly called the *umDoni* (Zulu) tree, is found throughout South Africa. The tree is utilised for its fruit, bark and wood by many villagers, and this demand has placed potential pressure on existing populations. It is necessary to conserve this widely used tree before it becomes threatened by over-utilisation. Seeds of *S. cordatum* are recalcitrant and storable only in the short-term at 16^{o}C over moist paper towel (hydrated storage). The study was initiated to follow deterioration of the embryonic axes, in relation to dehydrated versus and hydrated storage. However, for electron microscopic investigations, it was crucial that material was properly fixed to obtain samples that accurately represented the *in vivo* conditions. This proved to be challenging, as explained below, and changed the original aim of the project.

The high phenolic content of *S. cordatum* seeds and axes makes fixation, using an aldehyde-based fixative, such as glutaraldehyde, difficult, as the aldehyde groups bind to phenolic compounds, forming large oligomers that tear out during sectioning. This causes sections to become fragmented, making viewing with the transmission electron microscope (TEM) impossible. The quest to visualise the ultrastructure, consequently became an additional focus of the project.

Substituting glutaraldehyde with alternate primary fixatives including potassium permanganate ($KMnO_4$) and 1% osmium tetroxide (OsO_4) did not improve the situation. Cryo-fixation followed by freeze substitution was then attempted. Three substitution media, comprising glutaraldehyde, tannic acid, osmium tetroxide and acetone were used, all providing similar, unsatisfactory results showing ice crystal damage. Eventually, glutaraldehyde fixation was modified where samples were fixed in glutaraldehyde while being exposed to microwave energy. Results from this method of fixation were far better, with fine structure adequately preserved.

A second facet of the project was aimed at producing explants alternative to seed-derived zygotic axes. Cotyledonary explants used in an attempt to produce somatic embryos, were cultured onto media which incorporated various concentrations of 2,4-D, BAP and NAA. The callus produced was sub-cultured onto regeneration media, which included NAA and BAP or PGR-free media, did not develop further. Zygotic axes cultured onto shoot multiplication medium containing BAP and NAA produced adventitious shoots which produced roots when subcultured onto media containing GA_3.

PREFACE

The Experimental work described in this dissertation was carried out in the School of Biological and Conservation Sciences, University of KwaZulu-Natal, Westville from January 2006 to December 2008, under the supervision of Professors P Berjak and NW Pammenter and Dr James Wesley-Smith.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others, it is duly acknowledged in the text.

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The initial aim of this project was to assess the response of excised embryonic axes of *Syzygium cordatum* Hochst. seeds to dehydration. One of the analytical tools to be used in this investigation was transmission electron microscopy (TEM) which requires adequate fixation of the material. However, largely because of the presence of considerable quantities of phenolic compounds, considerable difficulty was experienced in fixing embryonic axes of *S. cordatum*. Consequently, the focus of the thesis shifted to include the development of protocols for preparing S. cordatum embryonic axes for TEM.

CHAPTER 1: INTRODUCTION

Genes code for key characteristics of all life, and it is this genetic diversity that is essential for the improvement or sustainability of crops (Hobbelink, 1991). The evolution of plants and the development of seeds (which reflect the genetic diversity) has allowed spermatophytes the means to disperse this diversity and inhabit new areas (Hartmann *et al.*, 2002). The preservation of seeds is probably the most widely used and effective method of conservation of genetic diversity (FAO, 1996), as seeds are capable of producing a seedling, when conditions are suitable, demonstrating their capacity for survival as regenerative structures (Copeland and McDonald, 2004). The correct storage of seeds is essential in ensuring their successful utilisation in agriculture, and apart from provision of food and feedstock, seeds are stored as base and active collections in order to facilitate long-term preservation of genetic biodiversity. However, seed deterioration during storage in seed banks and large commercial stores is a major problem, with losses in excess of 25% occurring annually due to breakage (i.e. physical damage) and microbial spoilage (McDonald and Nelson, 1986). Under ambient conditions, storage life spans are generally limited, but optimal storage conditions can greatly increase seed longevity, up to decades or even centuries (Beattie and Boswell, 1993).

1.1 Seed physiology

1.1.1 Orthodox vs. recalcitrant seeds

Seeds are assumed to be storable based on the premise that seeds show orthodox post-harvest or post-shedding behaviour (Roberts, 1973). Orthodox seeds acquire desiccation tolerance during

development, which can be attributed to two types of products (1) sugars and oligosaccharides and (2) specific proteins (Bewley and Black, 1994), together with a controlled shut-down of metabolism. Orthodox seeds of most species also undergo maturation drying on the parent plant i.e. they pass into a metabolically inactive state (Bewley and Black, 1994); thus orthodox seeds have good storage potential (Roberts 1973). This type of seed may be stored for predictable periods under defined conditions of temperature and seed water content without loss of seed quality, and the longevity of these seeds increases (within limits) logarithmically with decreasing water contents (Ellis and Roberts, 1980). For orthodox seeds, each percent increase in moisture content halves the lifespan of the seeds, and for every 5^{o}C decrease in temperature the lifespan of the seeds is doubled (Harrington 1972). In contrast to these long-lived seeds, recalcitrant seeds (Roberts, 1973) have surprisingly short life spans and few if any of the mechanisms that confer desiccation tolerance to orthodox seeds are present (Pammenter and Berjak, 1999). It is unlikely that these unpredictable seeds will conform to the 'rules' of storage i.e. low storage temperature and low relative humidity (Harrington, 1972). A number of crop and exploited wild species in Africa produce recalcitrant seeds, with *Syzygium cordatum* being one of them.

Recalcitrant seeds are produced by a range of monocotyledonos and dicotyledonous species (Berjak and Pammenter, 2004), and have been suggested to be the ancestral seed condition of spermatophytes (von Teichman and van Wyk, 1994; Pammenter and Berjak, 2000). However, contrary to this view, Dickie and Pritchard (2002) suggest that orthodoxy is the ancestral state in seed plants. Recalcitrant and orthodox seeds differ in their ecology, morphology and physiology (Chin *et al*, 1989; Daws *et a.l*, 2004). Most recalcitrant seeds, such as coconut, mango, avocado and cocoa, are from perennial trees of moist tropical provenances and most of the temperate species produce orthodox seeds. However, some oaks (Berjak and Pammenter, 2004) and citrus trees (Copeland and McDonald, 2004) which produce recalcitrant seeds may originate from temperate regions. It seems likely that recalcitrant seeds are produced predominantly in regions such as the moist tropics where the acquisition of desiccation tolerance would be of little advantage (Berjak and Pammenter, 2004). However, recalcitrant seeds are also produced by some species originating from dry environments (Berjak and Pammenter, 2004), such as *Boscia senegalensis* from the Sahelian zone (Danthu *et al*., 2000), and some of the amaryllids (Sershen *et al.*, 2008).

Most recalcitrant seeds reach maturity in their fruit (Chin and Roberts, 1980), which are covered by fleshy arilloid layers and an impermeable testa (Chin *et al*., 1989). At maturity, these seeds have a

much higher water content, and are generally larger than orthodox seeds (Copeland and McDonald, 2004). Orthodox seeds can be successfully dried to seed water contents of about 0.05 g g^{-1} without damage and are able to tolerate freezing temperatures (Berjak and Pammenter, 2004). Recalcitrant seeds on the other hand are desiccation sensitive and cannot be dried slowly to water contents below about 0.8 g g^{-1} without serious damage and are injured by freezing (Berjak and Pammenter, 2004; Copland and McDonald, 2004). The degree of desiccation tolerated by recalcitrant seeds of different species can vary considerably when dehydrated under similar conditions (Berjak and Pammenter, 2004). Interestingly, Daws *et al.* (2004) observed that larger seeds of *Vitellaria paradoxa* survived drying when compared to the smaller seeds. This was not a result of increased desiccation sensitivity, but rather the fact that the larger seeds took longer to dry, and were at a higher water content.

Although recalcitrant seeds are shed at high to very high water contents and are metabolically active at the time of shedding, the distinct variation in seed behaviour makes defining the nature of recalcitrance difficult (Berjak and Pammenter, 2004). Differences in response to desiccation can be attributed to size, structure, testa/pericarp and chemical makeup of the seed (Berjak and Pammenter, 2004). Recalcitrant seed metabolism continues from development to germination with little indication of when maturity is reached (Berjak *et al.,* 1989; Farrant *et al.,* 1989; Pammenter *et al.,* 1994), hence their developmental status is constantly changing (Berjak and Pammenter, 2004). For most recalcitrant seeds, desiccation sensitivity is greatest when metabolic rate is at its highest (Berjak and Pammenter, 2004). Recalcitrant seeds show increased desiccation sensitivity during germination (Berjak and Pammenter, 2004) because when they reach the stage of mitosis and cellular expansion by vacuolation during germination, exogenous water is required by the seeds (Farrant *et al*., 1986; Berjak *et al*., 1989; Pammenter *et al.*, 1994).

The response of recalcitrant seeds and their excised embryonic axes to drying is dependent on the rate at which water is lost (Normah *et al*., 1986; Pammenter *et al*., 1991; 1998; Pritchard, 1991; Kundu and Kachari, 2000; Potts and Lumpkin, 2000). The slow removal of water from material to levels above some lower limit, leads to the disruption of intracellular metabolism. The metabolically active cells may be inefficient in scavenging the toxic metabolic by-products that accumulate (Walters *et al*., 2001) and cause aqueous based oxidative reactions that are detrimental (Leprince *et al.,* 1990; 1993). This ‘metabolism-derived damage’ (Pammenter *et al*., 1998; Walters *et al*., 2001) is the suspected cause of lethal damage in slowly-dried recalcitrant seeds (Walters *et al*., 2001);

consequently, if drying is sufficiently rapid to levels above the lower limit of 0.45 - 0.25 g g^{-1} (Pammenter *et al.*, 1998), this damage does not accumulate to lethal levels. However, there is a limit to which an increase in drying rate will not prevent damage. Dehydration of material to below the lower limit, even if rapid, is thought to cause conformational changes in intracellular membranes and macromolecular structure as a result of the removal of non-freezable water, and this is known as 'desiccation damage *sensu stricto*' (Pammenter *et al*., 1998; Walters *et al*., 2001).

1.1.2 Difficulties associated with storage of recalcitrant seeds

Deterioration during storage of orthodox seeds is inevitable. This deterioration is irreversible and varies among seed populations and seed lots (Delouche, 1973), but under optimal conditions, it is possible to retard the rate of deterioration (Delouche, 1973). Relative humidity and storage temperature are crucial factors that affect longevity of both recalcitrant (Berjak and Pammenter, 2004) and orthodox (Harrington, 1972) seed storage. Other factors include the atmosphere of the storage environment that affects viability of seeds, especially for seeds with high water contents, as increased O_2 levels hasten loss of viability (Harrington, 1972), and in some cases high CO_2, N_2 or a vacuum may also lead to retarded growth (Harrington, 1972). However, the large size (morphology) and physiological characteristics of recalcitrant seeds also make storage difficult (Copeland and McDonald, 2004) even under the best possible conditions.

The starting quality of both recalcitrant and orthodox seeds is an important factor affecting the storability of seeds (Hong and Ellis, 1996; Pammenter and Berjak, 1999); thus it is imperative that seeds are harvested in the best possible condition to optimise storage (Berjak and Pammenter, 2004). Pre-storage management (and maintenance of high tissue water content in the case of recalcitrant seeds) also significantly affect the success of their short- to medium-term storage (Berjak and Pammenter, 2004). The retention of recalcitrant seed vigour and viability is also dependent on the elimination, or minimisation, of associated mycoflora (Berjak and Pammenter, 2004). Fungal contaminants may gain access to the seed tissue at any time from flowering to the post-shedding phase (Mycock and Berjak, 1992). Where possible, recalcitrant seeds or their fruit should be harvested from the parent plant rather than picked off the ground this poses an additional problem with the exposure of such seeds and fruit to soil microbes, in addition to any that they might harbour prior to shedding. If collected soon after shedding, fungal propagules may be restricted to the surface or periphery of the seeds, making eradication easier (Sutherland *et al.,*

2002); however, both orthodox and recalcitrant seeds may be infected by systemic transmission via the parent plant (Mycock and Berjak, 1992). Hydrated storage at ambient temperatures (Mycock and Berjak, 1990) and the high water content of recalcitrant seeds (Sutherland *et al.* 2002) provide ideal conditions for fungal proliferation, affording the mycelium the opportunity to become established deep within the seed tissues, making it virtually impossible to eradicate (Sutherland *et al.,* 2002). The active fungal metabolism during hydrated storage of recalcitrant seeds contributes to the considerable limitations on the storage life span and seed vigour and viability (Berjak, 1996; Calistru *et al*., 2000). Both degradation of the seed tissue by fungi and toxins produced by the fungi may be possible causes of seed deterioration during storage. Fungal respiration also produces metabolic water that can increase seed water content. The additional source of water could increase the metabolic activity of the seeds which could then show an increased rate of germination in storage if the metabolism of the seeds is able to outpace that of the fungal contaminants (Berjak and Pammenter, 2004).

Periodic aerosol applications of fungicide to seeds after removal of the pericarp have been shown to increase the short- to medium-term storage period of seeds by curtailing the fungal activity (Calistru *et al.*, 2000). Topical fungicides, such as Tebuconazole (triazole) found in Orius, are commonly used as a foliar or seed application, and are used as a protective or curative treatment by inhibiting the biosynthesis of ergosterol, an important component in the integrity of fungal cell membranes (http://edis.ifas.ufl.edu/PI105). However, if used as a curative treatment, application must be early as it is not effective against spores produced by the fungus (http://edis.ifas.ufl.edu/PI105). Triazoles are considered as a more potent fungicide treatment, and low concentrations of the active ingredient can inhibit shoot production (Clifford and Lenton, 1979; Kaufman and Song, 1988; Davis and Sankhla, 1988; Raghava and Raghava, 1998; Bisht *et al*., 2000). Azoxystrobin (strobilurin), found in Heritage, is a broad spectrum fungicide that acts against *Ascomycetes*, *Basidiomycetes*, *Deutoromycetes* and *Oomycetes* (http://www.pan-uk.org/pestnews/Actives/azoxystr.htm), and inhibits the electron transfer in mitochondria which interrupts energy production, preventing growth of fungi (http://en.wikipedia.org/wiki/Strobilurin). Fludioxonil (phenylpyrrole), found in Celeste 100 FS is also a broad spectrum fungicide used as a seed treatment that acts against the fungal pathogens *Ascomycetes*, *Basidiomycetes* and *Fungi imperfecti* by disrupting osmo-regulation.

The use of biological control agents such as strains of the *Trichoderma* genus found in Eco T and Eco 77 has also proven to be useful in the prevention of fungal proliferation. *Trichoderma* spp are

described as free living, avirulent, opportunistic fungi found in soil and root ecosystems. They are considered plant symbionts and parasites of other fungal species. Different strains of *Trichoderma* spp produce over 100 metabolites including those that are shown to have antibiotic properties or are extracellular proteins (Harman and Howell, 2004). *Trichoderma* spp also parasitise other fungi by growing towards target fungi and releasing fungitoxic enzymes capable of degrading cell walls and also peptaibol antibiotics (Harman and Howell, 2004). Holes can be produced in the target fungus, allowing for the direct entry of the *Trichoderma* hyphae into the lumen the target fungus (Harman and Howell, 2004). *Trichoderma* spp also compete for exudates from seeds that stimulate germination of pathogenic propagules in soil (Harman and Howell, 2004). Additionally, *Trichoderma* spp. can induce localised and systemic resistance of plants against pathogens (Harman *et al*., 2004). However, Pammenter *et al.* (1994) believe that it is the generation of highly destructive reactive oxygen species produced by ongoing metabolism in the seed tissues in the absence of additional water to be the most damaging, thus limiting longevity

If recalcitrant seeds are stored in air-tight bags and containers, the storage units should be opened and the seeds turned constantly to prevent toxic anoxic conditions building up (Berjak and Pammenter, 2004). Turning is potentially harmful as seeds may incur mechanical damage and the chance of micro-organisms being introduced into the mixture is increased (Berjak and Pammenter, 2004).

Studies on the short- to medium-term storage of recalcitrant seeds suggest that storage temperature must be kept as low as possible, while avoiding chilling injury (Berjak and Pammenter, 2004). There are many tropical species that produce seeds that show damage or are killed when stored at low (chilling) temperatures, and since many tropical seeds species are recalcitrant, storage of these seeds at sub-ambient temperatures does not increase viability (Chin and Roberts, 1980). However, storage at these temperatures could have several advantages: microbial growth and respiratory activity are decreased; the rate of cellular degradation is reduced; and temperatures of approximately $2^{o}C$ can prevent imbibed seeds germinating prematurely (Chin and Roberts, 1980). Boroughs and Hunter (1963), working with the recalcitrant seeds of *Theobroma cacao* L. suggested that the decline in viability of these seeds stored at lower temperatures can be attributed to three possible causes: (i) the cessation of a temperature dependent, rate limiting reaction that causes lethal metabolic disturbances (ii) the absence of protective substances found in seeds that are chilling tolerant, and (iii) cold-induced membrane permeability changes that allow the release of toxic

material. Other authors have suggested different reasons for the chilling damage response of recalcitrant seeds. Simon *et al.* (1976) proposed that seeds of *Cucumis sativus* L. cv. and *Phaseolus aureus* Roxb. show susceptibility to low temperatures due to protein denaturation, and Wolfe (1978) attributed chilling susceptibility to a decline in membrane fluidity, which alters the membrane thickness, permeability and electric field. Also, lower temperatures cause alterations in cation concentrations and ordering of water surrounding the membrane which causes conformational changes of membrane bound enzymes, thus affecting enzyme activity.

Additional to the loss of seeds through desiccation and chilling damage, and spoilage through micro-organisms, seed losses occur through germination in storage. Recalcitrant seeds, unlike orthodox seeds, are shed at water contents that permit germination (Chin and Roberts, 1980). Although low storage temperatures reduce the rate of germination in storage, when storing chilling susceptible seeds, loss of viability can occur through chilling damage (Chin and Roberts, 1980). However, germination inhibitors occurring naturally in seeds and fruit may prevent germination during storage of seeds, and thick seed coats of recalcitrant seeds may delay germination. The use of artificial chemical inhibitors such as methyl-1-naphthaleneacetate, although delaying germination, produces genetically deformed seedlings (Barton, 1965). Alternatively Pyke *et al.,* (1934) suggested that harvesting recalcitrant seeds such as *Theobroma cacao* before maturity may reduce germination in storage; however, Evans (1950) suggested that this produces irregular germination and seedlings that are prone to genetic abnormalities. Thus early harvesting should be investigated for individual species. The use of polyethylene glycol and sugar solutions as inhibitors seemed to prevent germination through osmotic inhibition (Chin and Roberts, 1980). Chin and Roberts (1980) suggest that recalcitrant seeds may be stored at high water contents slightly below the fully imbibed state, preventing germination with no loss of the physiological advantages associated with imbibed seeds. However, more recent work (Drew *et al.,* 2000) has shown that such sub-imbibed storage is actually detrimental as it is akin to a prolonged mild water stress.

1.1.3 *In situ* vs. *ex situ* conservation

Conservation of plant species can be divided into two categories, *in situ* and *ex situ*. *In situ* conservation is described as conservation within the confines of communities in their present and ideally, future state. Both domesticated and wild plants can be maintained in this manner, where they are subjected to a variety of selection pressures, and the environment is therefore described as

dynamic (Frankel *et al*., 1995). The aims of *in situ* conservation of forest genetic resources are to maintain economic production and replant with local seed sources (Frankel *et al.*, 1995). *Ex situ* conservation, in contrast, is dependent on support and management by humans (Frankel *et al.*, 1995). Most material for *ex situ* conservation is represented by seeds or vegetative tissues that are maintained in an artificial environment or whole plants in botanic gardens. Preserving an adequate representation of living plants is difficult, especially in the case of large, long-lived plants; thus there are several advantages to *ex situ* conservation, particularly as seeds. Seed conservation is relatively safe from environmental hazards and changing administrative policies; distribution is easier; little storage space is required compared with plantations and maintenance is inexpensive (Thompson, 1975). *In situ* conservation is closely linked to, and must compliment *ex situ* conservation, as *in situ* conservation of forests provides material for use or for conservation *ex situ*. Seeds in *ex situ* storage are sampled periodically and provide a source of germplasm material for research purposes. Additionally seed orchards can be established, that act as a reserve in case the need to replant the primary *in situ* population arises (Frankel *et al.*, 1995).

1.1.3.1 Methods of *ex situ* conservation

Gene banks, an example of *ex situ* conservation, assemble material that is made readily available to users, and also participate in long-term preservation of material (Frankel *et al.*, 1995). A Genetic Resources Centre has been proposed as the operational unit by the FAO panel of experts (FAO, 1973; Frankel, 1975). Such a centre consists of two parts: a base collection for long-term seed storage and an active collection used in the multiplication and regeneration of seeds. Active collection seeds are supplied to users and used in the documentation and evaluation of accessions in the base collection (Frankel *et al.*, 1995). When germplasm is collected, a list of plant characteristics, an accurate location and description of the location must be recorded to aid future scientists, plant breeders or germplasm specialists, before treatment for storage commences (Plucknett *et al.*, 1987). According to various authors, varying seed numbers are required to accurately represent the genetic diversity of a species, ranging from a few hundred to a few thousand (Plucknett *et al.*, 1987). Generally, gene banks store seeds at moisture contents between 3 and 7% and at temperatures between 5 and -20^{o}C, conditions which are suitable for orthodox seeds (Roos, 1989). However, in the case of recalcitrant seeds, which are desiccation and chilling sensitive (Roberts, 1973), other methods of storage need to be evaluated and employed.

1.1.3.2 Storage of recalcitrant seeds

Storage of such resources is generally *ex situ* as field gene banks, but this is not entirely fail-safe as plantings are at risk of damage by pests, disease and storms, and labour costs are high (Plucknett *et al.*, 1987). To overcome these threats, cryopreservation techniques or tissue culture methods may be used to store genetic information *in vitro* (Plucknett *et al.*, 1987). *In vitro* techniques have been developed for thousands of species for collecting, storing, manipulating and multiplying plant germplasm (Engelmann, 1997). There are several advantages to *in vitro* preservation; it is cost effective, not labour intensive and allows for the easy distribution and exchange of germplasm (Engelmann, 1997). Collections are also protected from exposure to insects, pests and diseases (Engelmann, 1997), making it a possible alternative to *ex situ* conservation as field gene banks.

An option for *in vitro* short- to medium-term storage is minimal growth storage, which utilises conditions that reduce growth (Engelmann, 1997). Traditionally, this has been used to maintain embryogenic callus (undifferentiated cells), shoot apices, plantlets, etc. *in vitro* (Engelmann, 1997). This is achieved by culturing material at low temperatures, in the presence of growth regulators or by using other less successful methods such as restricting nutrient or oxygen supply (Staritsky *et al.*, 1986; Withers, 1987a, b), and *ex vitro* seedlings may be kept in the shade to mimic conditions under natural canopies where light limitations restrict growth. However, slow growth is not often viewed as a long-term option, as loss of material is likely at each culturing (plating onto fresh medium) operation (Withers, 1988).

Long-term storage is essential in preserving genotypes (genetic resources) for plant breeders and scientists (Roos, 1989) and would be best achieved through cryopreservation (Engelmann, 1997) i.e. the storage of living material at temperatures near to or at the temperature of liquid nitrogen (-196^{o}C) (Withers, 1988). For orthodox seeds, cryopreservation has met with great success (Sakai and Noshiro, 1975), however for the more sensitive recalcitrant seeds, adjustments in moisture contents and thawing procedures are required (Frankel *et al.*, 1995). Additionally, recalcitrant seeds are generally too large for successful cryopreservation, thus a smaller explant must be used, and usually the most suitable is excised embryos or embryonic axes. Some cryopreservation techniques for germplasm storage have been successful (Englemann, 1997), and an advantage of cryogenically stored germplasm is that chromosome aberrations are at low frequencies (Frankel *et al.*, 1995),

renewal operations during suspended growth are eliminated and environmental hazards are reduced (Withers, 1988).

1.1.3.3 Events during freezing

The freezing point of cytoplasm is generally above -1^{o}C. However, cells can remain unfrozen at temperatures below -1^{o}C, which is known as supercooling. This can occur at temperatures as low as -10 to -15^{o}C (Mazur, 1970). The formation of ice crystals occur at nucleation sites and can occur extracellularly while the intracellular region remains supercooled (Mazur, 1963). This indicates that the cell membrane can prevent the growth of ice crystals into the supercooled cell interior and that cells neither contain nor are efficient nucleators of supercooled water (Mazur, 1970). Since the supercooled water has a vapour pressure higher than ice at -10 to -15^{o}C, equilibration between the interior and exterior can be established. Cells that are cooled slowly (<0.1^{o}C min^{-1}) or that have a high permeability to water will equilibrate by the outflow of supercooled water i.e. by dehydration; and cells cooled rapidly (>100^{o}C min^{-1}) or have a low permeability to water will equilibrate by intracellular freezing (Mazur, 1970). As cells lose liquid water, either by dehydration or freezing of intracellular water, they are exposed to a second set of physical-chemical-events (Mazur, 1970). The loss of water results in an increased concentration of intra and extracellular solutes that eventually precipitate as their solubilities are exceeded, ultimately altering the pH (Mazur, 1970). This was termed solution effects by Mazur (1970). The intracellular ice crystals produced by rapid freezing are usually small and have higher surface energies than larger ice crystals. These surface energies are reduced by growth of the small ice crystal or by fusing with other small ice crystals termed recrystallisation. Recrystallisation can occur during the solid frozen state and on warming, especially if warming is slow (Mazur, 1970).

1.1.3.4 Mechanisms of damage

Damage to cells during cooling and thawing can be attributed to both solution effects and to intracellular ice formation. Changes in pH through solution effects damage cells by causing membranes to become leaky during cooling and thawing (Mazur, 1970). Lovelock (1954) proposed that the increased electrolyte concentrations produced during freezing disrupts lipids in membranes, thus making the cells leaky. The cells experience osmotic shock during thawing as a result of an inflow of water. Levitt (1962) attributed freezing damage in higher plants to disulphide bonds

formed as a result of compaction of macromolecules by dehydration during freezing. However, Mazur (1970) speculates that freezing damage is a result of recrystallising ice crystals exerting sufficient force during the frozen state and on thawing, to rupture plasma and organelle membranes.

1.1.3.5 The use of cryoprotectants

In many instances, cryoprotectants are used as a method of minimising freezing damage. According to Lovelock (1954), cryoprotectants such as dimethyl sulphoxide (DMSO) and glycerol are a group of low molecular weight compounds that enter the cell and is effective by lowering the electrolyte concentration in the unfrozen, supercooled cell (Mazur, 1970). These cryoprotectants are described as penetrating and protect against solution effects produced during slow cooling (Mazur, 1970). The non-penetrating, high molecular weight cryoprotectants such as polyvinylpyrrolidone (PVP) draw water from the cell into the surrounding environment by forming an osmotic differential, thereby lowering the cell water content and reducing the amount of available water forming ice crystals (Mazur, 1970).

Axes of *S. cordatum* are far too large and have a w.c. of ≈ 2.0 g g^{-1} after flash drying (see later), which is far too high for cryopreservation (w.c. ≈ 0.3 – 0.4 g g^{-1}), hence a suitable alternate explant needs to be determined for cryopreservation.

1.2 Tissue culture

Tissue culture techniques reduce the parent plant to its basic constituents of cells, tissues and organs for study (White, 1954), and involves the use of various plant growth regulators in the culture medium in order to manipulate the structure and function of cell types. Other constituents such as activated charcoal, PEG 6000 and citric acid included in the culture medium are to reduce the effects of leached phenolic compounds. However, these inclusions did not show an improvement in survival of the cultures. Eventually, sub-culturing was done on a weekly basis for callus producing cultures to prevent the toxic effect of the accumulation of phenolic compounds.

An essential difference between animal and plant cells is that some plant cells are totipotent i.e. they possess the ability to divide and regenerate to form new, complete plants (Compton *et al.*, 2000) if provided with the correct external conditions (White, 1954), and if the cells of an organism are

totipotent, then cellular differences can be attributed to the influences of the microenvironment (White, 1954). *In vitro* plant tissues generate several types of primordia that eventually differentiate into various organs such as roots, shoots and embryos (Schwarz and Beaty, 2000). However, according to Dodds *et al.,* (1985), embryos are not classified as organs as they have no vascular attachments with the parent plant therefore for this section, somatic embryogenesis and organogenesis will be discussed separately.

1.2.1 Somatic embryos

The ability of plants to produce embryos is not limited to the development of a zygote during fertilization, as embryos can be induced to form from cultured plant tissues (Dodds *et al.*, 1985). The earliest demonstration that plants could be induced to produce non-zygotic or somatic embryos was published in 1958 by Stewart *et al.*, and in 1959, Reinert observed bi-polar embryos in root cultures of carrot after transfer from one medium to another, and since the earliest work, this phenomenon, known as somatic embryogenesis, has been observed in several higher plant species (Dodds *et al.*, 1985). However, for any plant species, there is only a certain range of explants that can be used to produce embryogenic cultures.

1.2.1.1 Somatic embryogenesis

Excised zygotic embryos (Gray, 2000) and embryogenic tissues such as hypocotyls and cotyledons from seeds can produce embryos without the intervening callus stage, when placed on an appropriate medium, which is commonly known as direct embryogenesis (Bewley and Black, 1994). Indirect embryogenesis is achieved from vegetative structures such as roots, shoot apices and young leaves of established plants that can be used as explants in the initiation of embryogenic cultures (Gray, 2000). An inoculation medium leads to the formation of a mass of undifferentiated cells (callus) and further manipulation stimulates redifferentiation of the cells with embryogenic potential to form embryos (Bewley and Black, 1994). Typical features of embryo-forming cells include large starchy granules, dense cytoplasm and a large nucleus with a dark nucleolus, and each embryo has to pass through the stages of embryo formation i.e. globular, heart and torpedo shaped for dicotyledons (Dodds *et al.*, 1985). To produce these embryogenic cells, the correct explant must be cultured into the appropriate medium with specific plant growth regulators (PGRs), namely auxins and cytokinins. Usually, the synthetic auxin 2,4-dichlorophenoxyacetic acid (2,4-D), is

added to the medium for most plant species. However other auxins such as indolebutyric acid (IBA), naphthoxyacetic acid (NOA), indoleacetic acid (IAA) and α-naphthaleneacetic acid (NAA) can be used (Gray, 2000). Auxins are thought to initiate differential gene activation to induce the formation of embryogenic cells, and through repetitive divisions, increases embryogenic cell populations. Additionally, auxins also prevent cell differentiation from embryogenic cells to embryos (Gray, 2000). However, in the case of pre-existing embryogenic cells such as immature zygotic cells, the use of auxins in the inoculation medium is not necessarily required (Gray, 2000). For an optimum response of the explant to the PGR, the PGR concentration must be ideal, as too low concentrations will not trigger a response and too high concentrations may be toxic to the explant. In addition to auxins, synthetic cytokinins such as 6-benzylaminopurine (BAP), kinetin and the natural cytokinin, zeatin, are used to induce embryogenesis (Gray, 2000). The transition of a non-embryogenic cell to an embryogenic cells occurs when a non embryogenic cell divides unequally to form a large cell and a smaller (embryogenic) cell (Gray, 2000). If the embryogenic cell continues to divide unevenly, a proembryonal complex is formed; however if the division is organised, a somatic embryo is produced.

1.2.1.2 Abnormalities of somatic embryos

Non-zygotic or somatic embryos are similar in structure to zygotic embryos, passing through the stages of embryo development and are easily identifiable (Gray, 2000). However, at full development, somatic embryos appear larger and wider set than zygotic embryos, presumably because developing cotyledons exert sufficient pressure onto developing zygotic embryos causing them to flatten (Gray, 2000). Somatic embryos growing in clumps develop asynchronously, and subjection to different culture media as the nutrient supply, is firstly diminished then replenished, during subsequent subcultures, thereby causing differences in development. This unregulated environment causes somatic embryos to prevaricate maturation, become disorganised and form new embryogenic callus, adding to the asynchrony. Also, extra cotyledons and poorly developed meristems are not uncommon (Gray, 2000).

1.2.1.3 Maturation of embryos

The final events of maturation of zygotic embryos are the accumulation of storage carbohydrates, proteins and lipids (Gray, 2000), decrease in water content and reduction or cessation of

metabolism. However in somatic embryos, there is a continuation of rapid growth often resulting in precocious germination. Although complete maturation is not necessary for obtaining plantlets, it is necessary for plantlet recovery. Several media incorporating ABA, PEG and high levels of sucrose have been shown to induce somatic embryo maturation (Gray, 2000).

Cotyledonary explants of *Syzygium cordatum* used in tissue culture experiments to generate somatic embryos produced a range of nodular and friable (i.e. delicate and loose) callus Fig 3.14 – 3.24). Friable callus can appear yellowish, green (Fig 3.21), white (Fig 3.22) or pigmented with anthocyanins (Dodds *et al.*, 1985). The inoculation media for cotyledonary explants contained the auxin 2,4-D which is recommended for non-zygotic explants, whereas the inoculation medium for the zygotic explant did not contain 2,4-D (Table 2.2). Callus production can be divided into 3 stages: induction, cell division and differentiation. During the induction stage, the explant cells prepare for cell division as cell metabolism is increased. The cell division stage is marked by a dedifferentiation of the divided cells and during the last stage, the cells differentiate. Areas in the dividing cells may produce primordia or meristemoids that produce roots, shoots or vascular nodules. Using a stereo microscope, closer examination of the range of callus produced by cotyledonary explants, revealed thin walled parenchyma (Fig 3.22) and vascular (Fig 3.23) cells. Although callus tissue has no predictable patterns of organisations, vascular differentiation in certain zones of the tissue has been observed in other plant species (Dodds *et al.*, 1985) and in *S. cordatum*, none of the above mentioned stages of somatic embryogenesis were identified in any of the callus cultures (Fig 3.13 – 3.21). The production of callus is also a response to wounding (Dodds *et al.*, 1985), thus the cut surfaces of the cotyledonary tissue provided an ideal surface for the formation and proliferation of callus tissue (Fig 3.13 – 3.21). The use of chlorophyll containing explants may produce callus containing chlorophyll (Dodds *et al.*, 1985) which may account for somatic embryogenesis Treatment 8 (Fig 3.20) producing green chlorophyll containing callus. The production of callus indicates that PGR levels were sufficiently high to evoke a response from the explants and were low enough to be non-toxic.

1.2.2 Organogenesis

The two principle events during *in vitro* micropropagation of plants via organogenesis is firstly to generate multiple shoot meristems that eventually produce microshoots, and secondly, once the microshoots have grown to an appropriate size, to induce production of *de novo* roots to form a

complete plantlet (Schwarz and Beaty, 2000). Factors stimulating organogenesis may include components of the culture medium, compounds produced by the culture and material carried by the explant from the parent plant (Dodds *et al.*, 1985).

As in somatic embryogenesis, during organogenesis *in vitro* and *in vivo*, organs such as roots and shoots arise from primordia, which differentiate into the various organs. In a dedifferentiation process, the explant cells undergo several divisions that eventually produce meristemoids, which are cells similar to meristematic tissue, however they are smaller, thin-walled and microvacuolated. These meristemoid cells are believed to be plastic in nature i.e. they have the ability to develop into various primordia and eventually organs.

Organogenesis, like somatic embryo production in plant tissue culture, can occur via two distinct pathways. The first is through direct organogenesis where the explant produces the organ without the proliferation of callus. In this case the explant dedifferentiates and produces the meristemoid cells, which then develop into the organ primordium. The second pathway is through indirect organogenesis, where the explant dedifferentiates to produce a mass of callus tissue that produces the meristemoids that develop into the organ primordium (Schwarz and Beaty, 2000). However, the production of callus increases the risk of somaclonal variation. The undifferentiated cells in the callus stage are prone to changes in the chromosomal structure, which can cause morphogenic and physiological changes in the organ produced. While some plants are more susceptible to this problem, the general rule is to avoid or minimise the callus producing stage (Schwarz and Beaty, 2000). Additionally, according to Dodds *et al.* (1985), continued sub-culturing of the callus may cause several changes in the tissue including hormone habituation, loss of friability and loss of organogenic potential (Thorpe, 1980).

During the dedifferentiation process, cells that respond to stimuli and produce meristemoids and primordia are described as competent. In the case of direct organogenesis, these may be individual cells or groups of cells that display competency and are believed to be the only cells that dedifferentiate and proceed to produce meristemoids, primordia and organs. In indirect organogenesis, callus cells may display this competency (Schwarz and Beaty, 2000). Once competency has been established and before organ primordium has been determined, the cells pass through an induction phase where Landauer (1958) suggested that a chemical and/or physical stimulus could modify a genetically determined developmental pathway, producing a mutant-like

phenotype termed a phenocopy. The end point of the induction phase is reached when the group of cells or the individual cells can be removed from the root or shoot induction medium and sub-cultured onto PGR free medium supplemented with minerals, vitamins and a carbon source, and proceed to produce the desired organs (Schwarz and Beaty, 2000). Again, the differentiation phase follows the induction phase and is the phase where development of the organ begins. (Schwarz and Beaty, 2000).

Damage to plant tissues can occur at any stage from harvesting to the different methods of storage. It is therefore necessary to understand the complex relationship between subcellular constituents to develop optimal methods of conservation. One technique used to enhance understanding is that of transmission electron microscopy (TEM). For this it is essential that material is adequately fixed to provide reliable ultrastructural information that accurately reflects the cell conditions. Existing fixation protocols are often unable to provide consistency in specimen fixation, thus it is necessary that suitable protocols are developed. For this reason, research into fixation of phenolic-rich tissue was incorporated into this project.

1.3 Transmission electron microscopy in the study of plant tissue

Plant material is composed of various cell types involved in an array of processes such as division, growth, differentiation and secretion, and with the help of transmission electron microscopy (TEM), the relationship between structure and function of these cells can be understood (Kiss, *et al.*, 1990). However, the electron beam in a transmission electron microscope (TEM) allows for only thin or ultrathin specimens to be viewed (Glauert, 1980), thus living tissue comprising mainly of water, proteins and ions must be stabilised before viewing (Kiss *et al*., 1990). This is achieved by fixing (killing) of the specimen (Glauert, 1980), usually with a chemical fixative, followed by dehydration via an organic solvent, such as alcohol or acetone, followed by embedding in resin and finally polymerisation (Kiss *et al*., 1990). Ultrathin sections (80 – 100 nm thick) are then cut, stained with contrasting heavy elements, and viewed using the TEM (Glauert, 1980).

1.3.1 Fixation

1.3.1.1 Artefacts of fixation

The rate of tissue death is primarily dependent on rate of penetration of the fixative into the tissue, thus low molecular weight fixatives, such as formaldehyde, are generally more effective in killing cells, but not necessarily in retaining ultrastructure. A direct relationship between rate of penetration and molecular weight does not always exist, as in the case of the fixative mercuric chloride ($HgCl_2$), which despite its high molecular weight, is actually quite a small molecule that penetrates tissue rapidly (Hayat, 1981). Ideally, fixatives should kill tissue promptly, while causing as little shrinkage or swelling of cells as possible (Glauert, 1980). According to this author, artifacts are nevertheless unavoidable, as the fixative changes the properties of the macromolecules of the cells. These changes, in turn, affect the interactions between the macromolecules and surrounding water and ions. This disruption causes water and ion shifts at the beginning of chemical fixation which may result in volume changes of the cell or its subcellular constituents (Edelman, 1989). Another possible consequence of chemical fixation is that soluble substances may be extracted from, or redistributed in, the cell (Edelman, 1989).

Chemical fixatives are not the sole cause of preparation artifacts, as the structural components of the plant cell such as the cell wall, large vacuoles and plastids, can each cause difficulties in every step of tissue preparation for viewing (Roland and Vian, 1991). The cellulosic cell wall, intracellular spaces and lacunae can act as barriers to fixatives and resins (Roland and Vian, 1991), resulting in poor fixation and infiltration, respectively. The large vacuole, characteristically found in plant cells, can comprise up to 80% of the cell volume and forms a large liquid-filled component. At the point of fixation, the vacuole can become leaky and the sometimes acidic content requires that buffers be adjusted accordingly (Roland and Vian, 1991). Such fixation responses of the vacuole produce a withdrawal of the tonoplast, giving the cell a plasmolysed appearance. A consequence of this plasmolysed appearance is an infolding of the tonoplast and cell membrane that may produce false impressions of endocytosis (Roland and Vian, 1991). Also, secondary metabolites such as phenols, polyphenols and tannins found in some vacuoles may produce substantial precipitates on fixation, resulting in poor tissue preservation. Plastids, at any level of differentiation, whether chloroplasts, chromoplasts or amyloplasts, pose a possible problem (Roland and Vian, 1991), as the lipids in the thylakoids in chloroplasts and lipid based pigments in chromoplasts may be extracted by alcohol,

and an accumulation of starch in amyloplasts may cause poor resin infiltration, leading to the risk of holes on the section (Roland and Vian, 1991).

1.3.1.2 Most common fixatives

Before the introduction of glutaraldehyde as a fixative by Sabatini *et al*. (1963), Luft (1956) suggested the use of potassium permanganate ($KMnO_4$) as a fixative alternative to osmium tetroxide (OsO_4), which became popular as a fixative for botanical specimens (Glauert, 1980). Permanganate fixatives were preferred for membranes as they provided good contrast against the surrounding cytoplasm (Glauert, 1980). It was later discovered that this clarity was achieved at the expense of the cell constituents as many, including soluble cytoplasmic proteins, ribosomes and RNA, were extracted (Glauert, 1980). However, according to Glauert (1980), although $KMnO_4$ is not the preferred fixative due to its inability to conserve all intracellular components, it is still used in some botanical studies as it is able to penetrate thick cell walls (van Steveninck, 1972).

Except for formaldehyde, the use of other aldehydes was a relatively late development in electron microscopy (Hayat, 1981). Formaldehyde, a mono-aldehyde, fixes tissue through the establishment of bridges between peptide chains by reacting with amino groups, thus forming a network. As a primary fixative, formaldehyde was originally found to yield poor results at the subcellular level due to large amounts of methanol, which is used to extend the shelf life of commercial formaldehyde solutions, acting as a coagulative fixative (Bowers and Maser, 1988). Paraformaldehyde, the solid polymer of formaldehyde, is used to produce methanol-free formaldehyde solution by dissolving the solid polymer in water, which improves results. This methanol-free form maintains the same functional aldehyde group (Bowers and Maser, 1988). Although formaldehyde penetration into tissue is rapid when compared with glutaraldehyde, its cross-linking reactions are slower than that of glutaraldehyde, making it a disadvantage for structural studies; additionally some cross-linking reactions of formaldehyde may be reversed during subsequent buffer incubations (Bowers and Maser, 1988).

Extensive studies by Sabatini *et al*. (1963), brought to light the usefulness of other aldehydes, and especially glutaraldehyde, to microscopists. The studies showed that primary fixation with glutaraldehyde followed by secondary fixation with osmium tetroxide (OsO_4), a strong oxidising agent, produced adequate preservation of fine structure in a variety of specimens (Hayat, 1981).

Glutaraldehyde, with two aldehyde groups has an increased potential for cross-linking peptide chains (Roland and Vian, 1991), including those of lipo- and glycoproteins, into a gel-like state (Bowers and Maser, 1988) and proved to be a far better fixative than the previously used single aldehyde, formaldehyde. The aldehyde group at either end of a 3 carbon flexible chain of glutaraldehyde allows cross-linking with numerous sites on protein molecules without distortion (Mercer and Birbeck, 1972); however, glutaraldehyde reacts poorly and sometimes not at all with lipids (Bowers and Maser, 1988).

Strangeways and Canti (1927) discovered that primary fixation with OsO_4 preserves delicate fine structure without alteration to mitochondria and fat droplets. Osmium tetroxide stabilises lipids by cross-linking or making them insoluble in organic solvents (Bowers and Maser, 1988). However rate of penetration of OsO_4 is slow and changes in tissue structure may occur before fixation is complete (Glauert, 1980). It is now generally used as a secondary or post fixative, after glutaraldehyde, to fix lipids not fixed by aldehydes (Glauert, 1980). Under these conditions, the slow rate of penetration of OsO_4 is not a disadvantage since the tissue has already been stabilised by glutaraldehyde (Glauert, 1980). However, prolonged exposure to OsO_4 is deleterious to samples. Since its earliest use as a fixative, OsO_4 has been used in part to fix proteins by cross-linking them; however, prolonged exposure to osmium tetroxide (OsO_4) makes them soluble by cleaving protein structures (Bowser and Maser, 1988). Maupin-Szamier and Pollard (1978) showed that OsO_4 biochemically cleaves protein (e.g. actin) and that reducing the temperature, pH and OsO_4 concentration, and substituting the cacodylate buffer with a phosphate buffer, decreased the rate of protein fragmentation; however, the extent of actin fragmentation remained unchanged given enough time (Bowers and Maser, 1988).

1.3.1.3 General characteristics affecting fixation

Apart from the chemical fixatives and cell constituents that affect the preservation of ultrastructure, there are several other contributing factors that may alter the quality of fixation. Among these are sample size, pH, temperature, duration of fixation and the method of application of the fixative (Hayat, 1981). To a large extent, the quality of fixation is related to uniformity of fixation throughout the tissue, as most fixatives fix tissue in layers. In the case of glutaraldehyde, the further into the tissue the fixative penetrates, the slower the rate of penetration to the center of the sample,

allowing for changes to occur within the tissue. Thus to achieve uniform fixation, it is necessary that sample size is as small as possible, irrespective of the tissue type or fixative used (Hayat, 1981).

Often the acidic conditions that develop during fixation precipitate both proteins and nucleic acids, which may be damaging to fine structure (Mercer and Birbeck, 1972). A decrease in tissue pH during fixation is usually an indication of cell death (Hayat, 1981). In the case of non-ionising fixatives such as glutaraldehyde and OsO_4, acidification may in part be explained by the interaction of the fixative with proteins. Protein macromolecules are irreversibly dissociated to low-molecular-weight proteins, resulting in an increase of ionisable carboxyl groups (Hayat, 1981). The addition of a buffer assists in preventing (Mercer and Birbeck, 1972) or reducing (Hayat, 1981) the change in pH, thus minimising tissue damage. Buffers are generally a weak acid or base with its accompanying salt, that resists the change in hydrogen ion concentration after the addition of small amounts of a strong base or acid (Hayat, 1981). Accordingly, the pH of the buffer is changed only slowly during fixation (Hayat, 1981).

Generally, smaller molecular weight fixatives penetrate tissue faster than higher molecular weight fixatives, but temperature plays an integral role in the rate of penetration (diffusion) of the fixative into the tissue. Higher temperatures (room temperature) increase the rate of diffusion as well as the rates of chemical reactions between cell constituents and fixatives (Hayat, 1981). However, higher temperatures and a longer fixation time cause excessive extraction of cellular material, whereas lower temperatures (4^oC) depolarise the plasma membrane and increase its resistance to ion permeation (Hayat, 1981), while reducing the extraction of cellular constituents and rate of degradation. A consequence is that rate of diffusion of the fixative is also reduced, allowing physiological changes to occur within the cell (Hayat, 1981). Once primary fixation is complete, proceeding at lower temperatures may be advantageous in reducing the extraction of any unstabilised constituents (Bowers and Maser, 1988). However, it must be noted that different fixatives and tissue types respond differently to temperature changes (Hayat, 1981). It is therefore essential that an optimal temperature for each fixative is determined to adequately fix tissue and minimise tissue damage (Hayat, 1981).

Duration of fixation is dependent on the factors that affect fixation. Optimum fixation time for most tissue types is not known, hence standard fixation times of 1-4 hours at 4^oC are used (Hayat, 1981). The specimen size and type, buffer, temperature and type of fixative contribute to the optimum

duration of fixation. In general, little is known about the effects of overfixation; however, obvious results of overfixation include extraction of cell constituents (Hayat, 1981).

1.3.1.4 Alternate methods of fixation

To prevent the extraction of important cell components during the fixation procedure, samples may be prepared at low temperatures (Glauert, 1980). Methods such as freeze-drying and freeze-substitution employ an initial freezing of the tissue. In freeze-drying, the tissue is frozen and the water removed under vacuum. Often substantial damage is caused to the freeze-dried tissue during embedding when the tissue is infiltrated with the liquid embedding medium (Glauert, 1980). To reduce this damage during infiltration, the method of so-called freeze-substitution was developed. Following rapid cooling of the tissue using liquid helium II at -269^{o}C, propane, propylene or Freon 22, the frozen tissue is left in a solution (i.e. the substitution medium usually consisting of organic solvents) at below 0^{o}C for extended periods (a few hours to two weeks), allowing the ice crystals that may have formed to dissolve slowly (usually in a dewar containing liquid nitrogen, i.e. at -196^{o}C), decreasing the chances of distorting the tissue. This is followed by infiltration of the tissue, usually at increasing temperature programs from -196^{o}C to room temperature. Since this method uses cryofixation, a cryoprotectant is usually used (Glauert, 1980). A major limitation of freeze substitution, however, is that specimens must be very small i.e. cubes with sides not exceeding 0.5 mm. This means that very restricted tissue segments can be used and, without preparing an inordinate number of blocks per specimen, microscopical observations may well be unrepresentative. Additionally, high pressure freezing (HPF) methods can be employed to freeze specimens 0.5 mm thick, with little ice crystal damage. Samples can then be freeze substituted, embedded in resin, sectioned and viewed with a conventional EM. Alternately, the frozen material can be fractured, coated in a freeze fracture apparatus and viewed in the frozen state with a cryo field emission EM (McDonald, 1999).

This alternate method of cryo-fixation, as described by Dubochet *et al*. (2007), fixes material in a ‘close-to native-state’ without the use of chemical fixatives, stains or aggregation, in a fully hydrated state. Cell water is vitrified and constituents maintained in an immobilised state. The specimen initially appears homogenous and contrast is apparent when viewing macromolecules that are arranged appropriately. However, there are disadvantages with preparation of this nature. The image presented is a 2 dimensional view of a 3 dimensional specimen and limitations occur when

constituents overlap (Dubochet, 2007). According to Matias *et al*. (2003), vitrification methods are also suitable for the preservation of small particles such as prokaryotes treated with a cryoprotectant. Specimen shape and cellular constituents are well represented.

Fixation protocols used in the preservation of plant material have generally been developed initially for animal tissues. Animal cells have a higher protein content than plant cells while plant cells are richer in carbohydrates. Although results for plant specimens are satisfactory, the differences in cell constituents between plant and animal cells increases the desirability of developing fixatives and fixation methods specific for plant cells (Hayat, 1981).

The accuracy reflected by fixed tissue relative to its former living state is the ultimate measure of quality of the fixation. However, for many plant species, there are considerable difficulties in preserving ultrastructure which implies minimum change from the living state. Among the problems besetting optimal fixation is the phenolic content of many plant cells, which causes major problems and frequently prevents cutting viewable sections. Therefore it is necessary to develop protocols to eliminate this problem, or at least, try to improve the quality of fixation.

1.3.2 Phenolics

1.3.2.1 What are phenolics?

Although animals, fungi and bacteria do produce phenolics, an estimated 80% of the known phenolic compounds are produced by plants (Rhodes, 1985). Originally, phenolics were considered so-called secondary compounds since they were thought non-essential in primary roles of life processes. However, studies have shown that these compounds are vital to many plants in a variety of ways (Vickery and Vickery, 1981). Phenolic compounds consist of a 6 carbon aromatic ring with one or more phenolic hydroxyl groups (Harborne, 1973; Rhodes, 1985). The simplest phenolic compound, phenol, consists of an aromatic ring with a single hydroxyl group. The aromatic nature of phenols allows for intense absorption in the UV region of the spectrum which is used in identification and quantitative analysis of phenols (Harborne, 1973).

Phenolics are generally water soluble and occur as glycosides in combination with sugars when found in vacuoles (Harborne, 1973). Within the group, the phenylpropanoids are the most widely

distributed, being found in nearly all higher plants (Rhodes, 1985). Other phenolic compounds include indole, hydroxylated quinines and quinoline and isoquinoline alkaloids that are found in a more limited number of plants (Rhodes, 1985). Phenylpropanoids exist as both monomers such as flavonoids and isoflavonoids, and polymers such as tannins and lignins, and are synthesised in specialised cells or organelles at various stages of organelle development. The phenolic hydroxyl group found in phenylpropanoids affects the chemical and physical properties of the compound by increasing the hydrophylic nature and conveying an acidic character to the molecule, which produces an array of colours. The hydroxyl groups also increase the chemical reactivity of the molecule, allowing it to bind to hydrogen intra-molecularly, or to other cell constituents such as proteins and alkaloids (Rhodes, 1985).

1.3.2.2 Synthesis of phenolics

There are two principle routes of phenolic synthesis, the shikimate pathway producing the phenylpropanoid nucleus, and the polyketide pathway which produces the A ring of flavonoids (Rhodes, 1985). There are several factors that activate phenolic biosynthesis, such as light quality, and factors such as infection, wounding and low temperature. Arguments have been put foward that secondary metabolites and phenolics are, in-fact, by-products of metabolism (Rhodes, 1985). However, these products are often more complex and toxic than their precursor, indicating that secondary metabolites and phenolic compounds are not merely waste products, but have specific roles (Rhodes, 1985). Phenylpropanoid production is regulated, occurs under specific conditions, and plants are equipped with a variety of enzymes capable of degrading phenolics. This is considered further evidence that these compounds are not simply by-products of metabolism (Rhodes, 1985).

1.3.2.3 Significance of phenolics

Simple phenols have strong antimicrobial properties and as a considerable proportion show some antibiotic action they could also possibly play a role in the way plants detoxify compounds (Rhodes, 1985). A typical response to injury or attack by fungi, bacteria, viruses and nematodes includes an increase in respiration, ethylene evolution and changes in phenylpropanoid metabolism. These reactions form part of the defence system of the plant, and can also be activated by abiotic factors such as heavy metals and other stress factors (Rhodes, 1985; www.organicashitaba.com/pc.html).

Apart from phenolics generated in response to such factors, plants may contain endogenous compounds which are present before infection, that prevent incursion of pathogens into the tissue (Rhodes, 1985). Injury can result in the activation of the phenylpropanoid biosynthetic pathway which forms a polymeric phenolic compound called 'wound lignin' (Rhodes, 1985). Its deposition in the cell wall is via the same route as normal lignin; however, it differs significantly from normal lignin in its monomeric constitution. Wound lignin is a result of an increase in extracellular phenolic compounds held in a polymeric form or as an ester bound material (Rhodes, 1985). The shikimate pathway also produces normal lignin which is a major component contributing to structural stability in many plant species (www.organicashitaba.com/pc.html).

Several pigments, many of which are flavonoids, are responsible for the array of colours that are used to attract both insect and bird pollinators (Vickery and Vickery, 1981). Pigments such as anthocyanins, which are responsible for red and blue colours, can function as honey guides, leading the insect pollinator to the nectaries and sexual structures (Rhodes, 1985). Apart from the colour that phenolics provide to attract pollinators, other phenolics such as vanillin, may act as a scent attractant (Rhodes, 1985). Phenolic compounds also react with taste buds producing different taste sensations (www.organicashitaba.com/pc.html); astringency from tannins may act as a feeding deterrent, bitterness from naringenin and pungency from capsaisin (Rhodes, 1985), while other phenolics may act as feeding attractants (www.organicashitaba.com/pc.html). Toxic compounds such as alkaloids and cyanogenic glycosides, not only have a bitter taste, which deters animals from feeding on the plant tissues, but may prevent further predation by actually killing the animal (Vickery and Vickery, 1981). Phenolics can also assist in reducing inter-plant competition for a particular environment by releasing phytotoxic compounds, either through diffusion from roots, or leaching from aerial parts of the plant into the soil. If maintained in high enough concentration, growth of competing plants can be prevented, a phenomenon known as allelopathy (Rhodes, 1985).

Phenolics are considered essential to the survival of plants. They provide protection from animals including insects through different taste sensations (Rhodes, 1985) and toxins (Vickery and Vickery, 1981), and their bright colours attract pollinators and seed dispersal agents. According to Rhodes (1985), phenolic compounds form an important part of the life processes of many plants, accounting for the considerable content of these compounds in particular species. *Syzygium cordatum* (Myrtaceae), the subject of the current study, is one such species.

1.3.2.4 Aldehyde – Phenol reactions

When phenolic laden material is fixed with an aldehyde fixative, large oligomers comprising hydroxymethyl phenols are formed (http://en.wikipedia.org/wiki/Phenol_formaldehyde_resin). In processing phenolic-rich material for TEM investigations, these large oligomers result in poor infiltration by the resin polymer, which results in poor section quality in the subsequent ultrathin sections. The hydroxymethyl groups are formed by the phenol formaldehyde reaction where the aldehyde may bind with the phenol at one of three possible sites on the 6-carbon aromatic ring. Each aldehyde can also react with up to two phenols, forming one large molecule (http://en.wikipedia.org/wiki/Phenol_formaldehyde_resin).

1.4 Aims

The aims of this project fall into two categories: firstly, studies on the recalcitrant nature of seeds of *Syzygium cordatum*, and the potential for cryopreservation of explants of this species, and secondly, improving the processing technique which will enhance future studies of the material.

Specifically, in terms of studies on the recalcitrant nature of the seeds, the aims were to:

- Determine whether seeds of *Syzygium cordatum* are recalcitrant and their response to drying and storage
- Develop optimum methods for micropropagation of *S. cordatum* by inducing somatic embryogenesis using various explants
- Assess the potential for drying and cryopreservation of these somatic embryos

The additional aim was to:

- Develop adequate methods for fixation of embryonic axes for TEM

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials: *Syzygium cordatum*

2.1.1 Distribution

Syzygium cordatum Hochst., commonly called the water berry tree or *umDoni* (Zulu), belongs to the Myrtaceae family which is well represented with over 3000 species in the southern hemisphere (Johnson and Briggs, 1981). In South Africa, myrtaceous species form an integral part of the indigenous flora, with *S. cordatum* as one of the more widely distributed species (Palgrave, 1977). *Syzygium cordatum* grows along water courses, wooded grasslands and forests (Pooley, 2003), in low-lands as well as medium to high lands (www.worldagroforestry.org). Distribution of the species is from Angola and Kenya, extending southwards to KwaZulu-Natal and Eastern Cape in South Africa (Codd, 1951). The trees are used as indicators of underground water, and in KwaZulu-Natal, as an indicator of land suitable for sugarcane farming (www.worldagroforestry.org). *Syzygium cordatum* trees can remain water-logged for several days without ill effects, and thereby are important in preventing the erosion of riverbanks (www.worldagroforestry.org). The tree is impervious to fire and withstands cold, but is damaged by frost (www.worldagroforestry.org).

2.1.2. Description

Syzygium cordatum is considered a multipurpose tree being planted or conserved for commercial, household or land improvement purposes (Hines and Eckman, 1993). It is a medium-sized tree that grows to a maximum height of 20 m. In older trees, the bark is dark brown to reddish and can be pulled off in cork-like square pieces (www.worldagroforestry.org), while the leaves are opposite, sessile, arranged at right angles, leathery and oval-oblong (Codd, 1951). The upper surface of leaves is bluish grey green while the under surface is a paler green (www.worldagroforestry.org). Whitish flowers bloom from spring to winter, yielding abundant nectar (Francis, 1977). The ovoid fruits are ≈2 cm long, and are fleshy berries that are green when immature and purple to purple-black when mature (www.worldagroforestry.org). Fruits contain substantial levels of phenolic compounds, particularly tannins, (Ndhlala, *et al.*, 2007) that constitute a defence mechanism against animals including insects. The seeds are polyembryonic with up to five embryos per seed.

2.1.3 Uses of *Syzygium cordatum*

The fruit is consumed by animals (that act as dispersal agents for the seeds) and humans (www.worldagroforestry.org), thus providing a source of nutrition, supplying vitamins and minerals in the absence of other cultivated fruit and vegetables (Hines and Eckman, 1993). Wild game such as kudu, nyala and bushbuck browse on the leaves. When boiled, the fruit is used to make an alcoholic beverage (www.worldagroforestry.org). A bluish-black dye is extracted from the bark or fruit by boiling and then fixing with lemon juice and salt (Hines and Eckman, 1993). The bark burns with a low odour and without excessive smoke, making the bark a good source for charcoal and firewood (www.worldagroforestry.org). Wood from *S. cordatum* that is water seasoned or water cured to increase resilience is useful in boat making (Codd, 1951) and its high density makes it ideal for furniture making. The bark can also be ground to a fine powder to make a fish poison used for fishing in small ponds (van Wyk *et al.,* 1997). In traditional African medicine, the roots and bark are boiled to produce a concoction that is used to treat indigestion and giddiness and the leaves, bark and roots are used in the treatment of tuberculosis, diarrhoea, and stomach and respiratory complaints (van Wyk *et al.,* 1997).

When *S. cordatum* populations are in close proximity to *S. guineense* and *S. gerrardii*, it apparently hybridises freely with the latter species (www.worldagroforestry.org). Propagation is usually by seeds which show 90% germination 25 d after sowing (www.worldagroforestry.org). Common pathogenic, opportunistic fungi of *S. cordatum* are *Botryosphaeria* spp. (Pavlic *et al.*, 2004) and according to those authors, symptoms associated with infection by this fungal genus include canker, dieback and in severe cases, tree death (Davison & Tay, 1983; Webb, 1983; Sharma *et al.*, 1984; Shearer *et al.*, 1987; Smith *et al.*, 1994; 2001; Old & Davison, 2000; Roux *et al.*, 2000; 2001).

The high demand for *S. cordatum* places great pressure on existing populations (Hines and Eckman, 1993), decreasing seedling recruitment and diminishing the genepool. Human encroachment has forced the use of marginal lands for growing trees in many countries (Plucknett *et al*., 1987) and the recalcitrant nature of seeds makes conventional medium- to long-term storage impossible. These factors make it essential to determine optimum long-term *in vitro* storage conditions that require little space and are cost effective to ensure the production of high quality material for re-introduction into natural habitats.

2.2 Methods

2.2.1 Seed collection and initial treatment

Seeds of *S. cordatum* were generally harvested by cutting off fruit bearing twigs. Fast removal of the pericarp was necessary to increase viablility, as the fruit lose condition within 2-3 days of harvesting. A total of approximately 2000 fruit were collected between 9am and 11am from five trees within a local park and neighbouring property. In the laboratory, the fruit were washed and separated into ripe and green fruit. Green fruit were surface dried and stored temporarily with the pericarp intact, in trays on dry paper towel at 16^{o}C. Immature seeds from green fruits were used in later treatments as they did not deteriorate as rapidly as ripe seeds. The pericarp/pulp was removed manually from ripe seeds, ensuring that the testa remained intact. Seeds were washed and rubbed vigourously with a rough piece of shade netting to remove any remaining pulp residue. Surface decontamination was achieved by soaking seeds in a 1% sodium hypochlorite solution for 10 min, followed by rinsing with sterile distilled water for 10 min and used for the assessments described. Seeds that were not used immediately were stored for a maximum period of 3 days at 16^{o}C in 300 x 600 mm trays lined with paper towel slightly moistened with distilled water. The seeds were covered with a thick layer of dry paper towel.

2.2.2 Physiological studies

This section of the project was to determine whether seeds of *S. cordatum* are recalcitrant and to determine their response to dehydration and storage.

2.2.2.1 Storage trial

Whole seeds were used immediately after surface decontamination and rinsing to determine optimum storage conditions for retention of vigour and viability, following soaking in a solution of chlorhexidine gluconate (2% concentration for 10 min), and a range of available fungicide treatments (see below). Two methods of storage were evaluated: (i) storage in a saturated atmosphere, in which seeds were suspended on a plastic grid above paper towel moistened with sterile distilled water, within sealed buckets, and (ii) storage at original seed water content sealed within plastic bags filled with vermiculite. The storage containers were kept at 16^{o}C and seeds sampled at two-week intervals until they were no longer viable. For assessment of the efficacy of

fungal treatments, following surface decontamination, seeds were divided into seven lots, a control, four fungicide treatments (Table 2.1) and two biological control treatments. The fungicide treatments consisted of a 2% chlorhexidine gluconate solution in combination with the fungicides (Table 2.1).

Table 2.1: Active ingredients and concentrations of fungicides used in storage trial of seeds of *S. cordatum*

Solution	Active ingredient and common name	Concentration of active ingredient
Control	----	----
Orius	Tebuconazole (triazole)	0.1 g l^{-1}
Heritage	Azoxystrobin (strobilurin)	0.4 g l^{-1}
Celeste 100 FS	Fludioxonil (phenylpyrrole)	0.33 g l^{-1}
Heritage and Celeste 100 FS	Azoxystrobin (strobilurin) Fludioxonil (phenylpyrrole)	0.4 g l^{-1} 0.33 g l^{-1}

Seeds were soaked in these solutions for 6 h while being agitated on an electric shaker, followed by drying in a lamina flow unit for 10 min to remove excess surface water, before being placed in their respective storage containers. The control was untreated after surface decontamination and agitated for the same period in distilled water. Additionally, two strains of the fungal genus, *Trichoderma* (ECO T and Eco 77), were used as biological control agents, in combination with the chlorhexidine gluconate solution to prevent the growth of pathogens. For the Eco T treatment, the seeds were initially maintained in the chlorhexidine gluconate solution for approximately 5 h. Exactly 1 g l^{-1} of the Eco T powder was added to a 1% carboxymethylcellulose (CMC) solution and mixed. The seeds were removed from the chlorhexidine gluconate solution and soaked for 30 min in the Eco T/CMC solution, removed, dried and stored as the other treatments. The procedure was repeated for the last batch of seeds using Eco 77 inoculum.

Samples comprising approximately 150 seeds from each treatment were stored in 3 containers with approximately 50 seeds in each container for all 7 treatments for each storage condition. For storage at the original seed water content, each sample of 150 seeds from each treatment were placed evenly in 3 separate, dry 305 x 270 mm Ziploc® bags with 250 ml vermiculite (Table: 2.2). For storage in a saturated atmosphere, the 150 seeds per sample from each treatment were separated evenly and placed in 3 sealed containers measuring 235 mm in diameter and with a depth of 180 mm

(Table: 2.3). The containers and lids were first sterilised with a 1% sodium hypochlorite solution and the seeds spread out on plastic grids which were similarly sterilised. Paper towel moistened with distilled water was placed at the bottom of the container, below the plastic grids, and dry paper towel was attached to the inside surface of the lids to prevent liquid condensate dripping onto the seeds. This was regularly monitored and changed during the course of the storage period. Throughout this study, seeds were stored in a laboratory seed store at 16^{o}C by this method.

Table 2.2 Number of seeds and replicates for storage of seeds treated with different fungicides and stored at original seed water content

Replicate	Control	Orius	Heritage	Celeste	Heritage + Celeste	Eco T	Eco 77	
1	50	50	50	50	50	50	50	Number of seeds
2	50	50	50	50	50	50	50	
3	50	50	50	50	50	50	50	

Table 2.3: Number of seeds and replicates for storage of seeds treated with different fungicides and stored at saturated atmosphere

Replicate	Control	Orius	Heritage	Celeste	Heritage + Celeste	Eco T	Eco 77	
1	50	50	50	50	50	50	50	Number of seeds
2	50	50	50	50	50	50	50	
3	50	50	50	50	50	50	50	

Water contents of seeds were analysed using the statistical program, SPSS 15.0, to perform a univariant analysis of variance with two fixed factors, storage regime (i.e. at saturated atmosphere and original water content) and fungicide treatment, to determine differences between storage regime and fungicide treatment. This was followed by a Tukey's test to determine a mean separation.

2.2.2.2 Dehydration of whole seeds

Twenty freshly collected whole seeds, immediately after collection, with the pulp/preicarp removed, were placed in each of five 305 x 270 mm Ziploc® bags, each containing 500 ml activated silica gel.

The initial weight of the 20 seeds in each bag was recorded and the seeds were left at room temperature to dehydrate until bags 1 – 5 had lost 5, 10 20, 30, or 40% water initially present, respectively. The control was from seeds that were not stored or dehydrated. Seeds weight was reassessed regularly to determine the amount of water loss by subtracting the dry weight from the original weight. Once the approximate amount of water was lost from each bag, axes were excised, ten of which were used to determine axis water content and the remaining ten axes were used to determine germination *in vitro* on full strength MS medium.

2.2.2.4 Hydration of whole seeds

This was done to estimate the period necessary for seeds to absorb 10% of its own mass in order to facilitate the uptake of the fungicide and bactericide solution. The rate of uptake of water by whole seeds was assessed over an hour time course. Axes were excised for water content and germinability measures.

2.2.2.2 Flash (rapid) drying of excised axes

Axes used in the flash drying trial were excised from ripe seeds immediately after collection and cleaning, and were accumulated in a closed Petri dish on damp filter paper to prevent or reduce any uncontrolled water loss, until excision was completed. Axes were excised and placed on a wire mesh supported above a fan in a glass jar half-filled with silica gel (illustrated in Pammenter *et al.,* 2002) and dried for 0, 30, 60, 120 and 180 mins. Ten axes from each drying time were used to determine water content, and the remaining 20 were allowed to re-hydrate for 30 min in a 1 μM $CaCl_2$ and 1 mM $MgCl_2$ solution (Berjak and Mycock, 2004). Axes were then surface decontaminated for 6 min in a 1% sodium hypochlorite solution, rinsed three times with distilled water under aseptic conditions and plated onto Petri plates containing a germination medium incorporating 30 g l^{-1} sucrose, 4.4 g l^{-1} (i.e. full strength) MS salts (Murashige and Skoog, 1962) and 8 g l^{-1} bacteriological agar in distilled water (pH 5.6 - 5.8). A maximum of 5 axes per Petri dish were plated in a manner similar to the seeds, then placed in the growth room with a temperature of 25°C and constant exposure to light intensity of 65 μmol m^{-2} sec^{-1}. Percentage germination, and root and shoot production were recorded. Axes were subcultured onto fresh medium on a weekly basis.

2.2.2.5 Water content determination

To determine axis water content for treatments, the axes were excised and the fresh mass of the samples was determined using an electronic balance, followed by drying at 70°C in an oven for approximately 48 h, after which dry mass was ascertained. To calculate the water content of axes on an individual basis, the following formula was used:

(Fresh mass – dry mass) / dry mass = water content. (g water g^{-1} dry mass)

2.2.2.6 Germination protocol

In vitro germination, using seeds from each storage treatment, was also carried out in conjunction with the water content assessments. The seeds were plated on a water agar medium incorporating 8 g l^{-1} bacteriological agar in distilled water (pH 5.6 - 5.8). Whole seeds were surfaced decontaminated for 10 min with a 1% sodium hypochlorite solution and rinsed three times with sterile distilled water in a lamina flow unit. Seeds were gently pushed into the medium until below the medium surface. A maximum of 2 seeds were plated in each germination container (500 ml jars), which was then placed in a growth room with a temperature of 25°C and constant exposure to light intensity of 65 µmol m^{-2} sec^{-1}. Germinating seeds were not sub-cultured.

2.2.3 Tissue Culture

This section of the project focuses on developing methods of micropropagation by inducing somatic embryogenesis and organogenesis using various explants and to determine if somatic embryos are explants suitable for cryopreservation.

2.2.3.1 Preparation of material

Cotyledons were cut into circular discs 1mm thick in a solution of 0.75 mg l^{-1} citric acid in a Petri dish. The cotyledon slices were kept in the citric acid solution until excision was completed, then surface decontaminated for 10 min by soaking in a 1% sodium hypochlorite solution followed by rinsing three times with sterile distilled water in a lamina flow cabinet.

Axes for tissue culture were excised in a solution of 0.75 mg l^{-1} citric acid and stored on moist filter paper in a Petri dish until excision was completed. Surface decontamination was carried out using the same technique as described for cotyledons above; however decontamination time was reduced to 6 min. Leaf explants were taken from plantlets grown *in vitro*, as leaves taken from the trees from which the seeds were harvested, showed contamination during culturing. Decontamination time was also reduced to 6 min.

The axes and cotyledon segments leached phenolic compounds into the medium which ultimately caused tissue death. To prevent large quantities of these compounds building up in the interim, the following methods were evaluated:

1. 0.75 mg l^{-1} citric acid was introduced into the medium to reduce the production of phenolic compounds (concentrations of 0.75, 7.5 and 75 mg l^{-1} citric acid were used for TEM fixation of embryonic axes; however, no improvement was noted with increased concentrations, thus 0.75 mg l^{-1} was used throughout.)
2. 1 g l^{-1} polyethylene glycol 6000 (PEG) was added to the medium to absorb the phenolics
3. 4 g l^{-1} activated charcoal was added to the medium to absorb phenolics
4. the amount of bacteriological agar added to the medium was reduced from 8 g l^{-1} to 7 g l^{-1} to facilitate diffusion of the phenolics away from the explant

However no improvement in survival of the explants was noted with these measures. Eventually, axes and cotyledons were sub-cultured once a week, instead of the usual once every 4 – 6 weeks, to prevent accumulation of phenolics. Adoption of these precautions created other problems:

1. the frequent sub-culturing increased contamination
2. sub-culturing once a week was time consuming and impractical
3. risk of mechanical damage with forceps to explants increased

2.2.3.2 Preparation of media for tissue culture

Several media incorporating MS salts (Murashige and Skoog 1962), sucrose, 7g l^{-1} bacteriological agar and plant growth regulators (PGRs) (Table 2.4 – 2.7) were prepared, autoclaved and poured into Petri plates or transferred into culture bottles. All cultures were maintained in a growth room at a temperature of 25°C and constant exposure to light intensity of approximately 65 µmol m^{-2} sec^{-1}.

2.2.3.3 Somatic embryo media

Table 2.4: Plant growth regulators, concentrations and explants used to induce somatic embryogenesis in embryonic axes or cotyledon slices of *S. cordatum* (for abbreviations, see pxiii)

Treatment	MS Strength	2,4-D (mg l^{-1})	Sucrose (g l^{-1})	BAP (mg l^{-1})	NAA (mg l^{-1})	Explant type
1	1*	0.5	30	0	0	Cotyledon
2	1	1	30	0	0	Cotyledon
3	1	1	103	0	0	Cotyledon
4	½**	0.1	30	0	0	Cotyledon
5	1	2	30	0	0	Cotyledon
6	1	3	30	0	0	Cotyledon
7	1	1	30	0.01	0	Cotyledon
8	1	1	30	0.1	0	Cotyledon
9	1	0	30	0.5	1	Cotyledon/Axes

*Full strength MS medium (1) = 4.4 g l^{-1} Treatment 9 included 10% (v/v) coconut water

**Half strength MS medium (½) = 2.2 g l^{-1}

Explants were cultured onto media promoting somatic embryo production (Table 2.4). Results, including change in tissue (i.e. development of callus) and contamination, were recorded every Monday, Wednesday and Friday. Explants were cultured onto fresh medium on a weekly basis. Nodules produced on explants were excised using a sterile dissecting needle and sub-cultured onto shooting and rooting media described in Table 2.5.

2.2.3.4 Shooting media

Table 2.5: Plant growth regulators, concentrations and explants used to induce shoot formation in leaves, nodules and embryonic axes of *S. cordatum*

Treatment	BAP (mg l^{-1})	NAA (mg l^{-1})	MS strength	Explant type
1	0.75	0	1	Leaves/Nodules
2	0.75	0	½	Leaves
3	1	0.5	1	Axes/Nodules

Leaf and embryonic axis explants were cultured directly onto the shooting media (Table 2.5) and were sub-cultured onto fresh medium on a weekly basis (using the same technique as above) until changes in the tissue were noted. Nodules that were produced by cotyledonary explants used in somatic embryo production (section 2.2.3.3) were excised and sub-cultured onto shooting media (Table 2.5). Sub-culturing onto fresh medium was done every 4 weeks rather than on a weekly basis. Media promoting shoot elongation were then tested using cultures producing shoots of various lengths.

2.2.3.5 Shoot elongation media

Table 2.6: Plant growth regulators and concentrations used to induce shoot elongation of adventitious shoots of *S. cordatum*

Treatment	BAP (mg l^{-1})	NAA (mg l^{-1})	MS strength
1	1	0.5	1
2	0	0	½
3	0	0	1

Media were prepared and autoclaved in 100 ml culture bottles. Petri dishes containing the same media were also prepared. Whole embryonic axis explants producing 3 - 4 mm shoots on media promoting shoot production (from section 2.2.3.4) were sub-cultured onto shoot elongation media (Table 2.6) in 100 ml culture bottles and 90 mm Petri dishes. Sub-culturing onto fresh medium was done every 4 weeks.

2.2.3.6 Rooting media

Table 2.7: Plant growth regulators, concentrations and explants used to induce root formation of elongated shoots of *S. cordatum*

Treatment	BAP (mg l^{-1})	NAA (mg l^{-1})	GA_3 (mg l^{-1})	Explant type
1	0	3	0	Shoots/Nodules
2	0.5	1	0	Shoots/Nodules
3	0	0	0.1	Shoots/Nodules
4	0	0	0.2	Shoots/Nodules
5	0	0	0.5	Shoots/Nodules

Media promoting root formation (Table 2.7) were prepared and dispensed into 100 ml culture bottles, then autoclaved. Shoots, approximately 20 mm tall, were excised from cultures under aseptic conditions, and gently pushed 3-4 mm into the medium in an upright position. Culturing onto fresh media was done every 4 weeks until roots developed. Results were recorded every alternate day.

2.2.3.7 Image capture

Cultures in Petri plates were viewed using a Nikon AZ 100 stereo microscope and images captured using NIS Elements D 3.0. imaging software. Culture bottles were photographed using a Nikon Coolpix 100 camera. Callus produced by explants was excised, placed in a drop of distilled water on a slide and squashed using a coverslip. Slides were then viewed using a Nikon DXM 1200 C light microscope and images captured using NIS Elements D 3.0 imaging software.

2.2.4 Processing for light and transmission electron microscopy

2.2.4.1 Glutaraldehyde fixation

For light (LM) and TEM fixation, axes were excised in a 0.75 mg l^{-1} citric acid using a surgical steel blade and fixed for 24 h at 4^{o}C in a glass, No 1 pill vial (10 mm diameter x 50 mm height) containing 2 ml 2.5% glutaraldehyde incorporating 1% caffeine in 0.1 M phosphate buffer (pH 7.2). The axes were then rinsed by immersion in the phosphate buffer (3 x 5 min), then post-fixed with 0.5% aqueous (OsO_4) for 1 h. Following post-fixation and further rinsing in the phosphate buffer (3 x 5 min), the samples were dehydrated through an increasing acetone series by immersing in 30%, 50%, 70% and 100% acetone (3 x 10 min) each. The samples were infiltrated in equal parts of Spurr's resin (Spurr, 1969) and 100% acetone for 4 h on a rotating mixer (10 rpm) to ensure even infiltration. Samples were then embedded in pure resin and left for 24 h on the rotating mixer to ensure full infiltration by the resin. The axes were removed from the resin and orientated in moulds filled with fresh pure resin, then polymerised in an oven at 70°C for 8 h. Semi-thin sections were cut using a Reichert-Jung Ultracut E microtome with a glass knife. Sections were picked up on a glass bead and placed on a drop of distilled water on a glass slide, dried and the section then stained with 1 % Toluidine Blue stain for 30 s. Prior to viewing, the sections were mounted in Permount Toluene Solution SP15-100 (Electron Microscopy Sciences, PA, USA) and coverslipped. The

sections were viewed using a Nikon DXM 12000C light microscope and images were captured using NIS Elements D 3.0 imaging software. Attempts were made to obtain ultrathin sections for TEM, but the material fragmented, due to the high phenolic content of the axes and poor fixation, thus sections could not be picked up. For this reason, trials employing improved methods of fixation were subsequently undertaken.

To improve TEM preparation, root and shoot meristematic tissue was separated to reduce sample size, and soaked for 10 min in 0.75 mg l^{-1} citric acid then fixed using the standard TEM protocol. However no improvement was noted after sectioning, soaking time was increased to 20 then 30 min and citric acid concentration increased to 7.5 and 75 mg l^{-1}. Positive results were achieved only once using 75 mg l^{-1} citric acid; however, this was inconsistent as only a small percentage of material fixed provided positive results. The root meristem was trimmed down further to 1 x 1 x 1 mm and fixed using the conventional fixation method with no improvement. To improve infiltration and embedding, Spurr's resin was replaced with the less viscous London Resin white (LR white) and the samples were dehydrated through an ethanol series by immersing in 30%, 50%, 70% and 100% ethanol (3 x 10 min) each. The ethanol was replaced with increasing concentrations of London Resin white at 4^{o}C in a refrigerator using the following method:

25% resin + 75% ethanol (2 x 15 min)

50% resin + 50% ethanol (2 x 15 min)

75% resin + 25% ethanol (2 x 15 min)

Samples were embedded in plain resin for 24 h at 4^{o}C. Each tissue sample was placed in a size 3 gelatin capsule (approximately 0.4 x 1.2 mm), filled with whole resin, sealed and polymerised at 60^{o}C for 24 h. A small hole was made at the end of the capsule to release air bubbles during polymerisation. Sections were cut as above; however, results did not improve.

Subsequently, less conventional methods of fixation such as using potassium permanganate ($KMnO_4$) and 1% osmium tetroxide as primary fixatives, and cryofixation followed by freeze substitution with different substitution media, were used.

2.2.4.2 Permanganate fixation

A 1.2% $KMnO_4$ stock solution (which becomes reduced in light) was prepared in a dark glass bottle. A veronal-acetate stock solution incorporating 28.9 g l^{-1} sodium veronal (barbitone sodium)

and 11.5 g l^{-1} sodium acetate (anhydrous) or alternatively 19.0 g l^{-1} sodium acetate (hydrated) in distilled water, was used as a buffer. The fixative comprised of 12.5 ml KMnO4 stock solution, 5 ml veronal-acetate stock solution and 2.5 ml distilled water, combined in a brown glass bottle. The pH was adjusted to pH 7.2 - 7.6 using approximately 5 ml 1N HCl. The final concentration of $KMnO_4$ attained was 0.6%. Root meristems were trimmed down to 1 x 1 x 1 mm in 0.75 mg l^{-1} citric acid solution, dabbed dry on filter paper to remove excess citric acid solution and placed in glass, No 1 pill vials. Approximately 2 ml fixative was added to each vial and the samples were fixed at 0^oC or 4^oC for 1, 2, 4, 6 and 12 h. Axes were subsequently dehydrated through a graded series of ethanol, infiltrated and embedded in LR white resin using the prescribed protocol. Semi-thin sections were cut using a Reichert-Jung Ultracut E and a glass knife, and prepared as described previously. Fixation and infiltration remained poor and did not allow ultrathin sections to be cut.

2.2.4.3 Osmium tetroxide fixation

Axes were excised and trimmed down to 1 x 1 x 1 mm in a 0.75 mg l^{-1} citric acid solution, dabbed dry on filter paper and fixed in 1% aqueous OsO_4 for 2 h, then rinsed by immersion into phosphate buffer (pH 7.2 for x 5min), dehydrated in a graded ethanol series, infiltrated and embedded with LR white resin. Semi-thin sections were cut using the Reichert-Jung Ultracut E microtome and slides prepared as above.

2.2.4.4 Freeze substitution

The axes for each treatment were excised and root meristematic tissue samples were trimmed down to 0.5 x 0.5 x 0.5 mm in 0.75 mg l^{-1} citric acid solution, dabbed dry on filter paper, and cryoprotected by soaking in a 30% polyvinylpyrridine (PVP) solution for 30 min. After dabbing on filter paper to remove excess PVP, samples were attached to the head of a steel pin and, using a Leica EMCPC cryo-preparation station set to standard settings, plunged into a tank containing liquid propane cooled to -183^oC with liquid nitrogen (LN), to flash freeze them. The flash frozen samples were immediately transferred to one of the pre-chilled substitution media (listed below):

1. 2% glutaraldehyde + 1% osmium + 2% tannic acid (to preserve microtubule structure) (aqueous)
2. 1% osmium + 2% tannic acid (aqueous)
3. 100% acetone

For each substitution medium, the samples were stored in the medium for 3 d at -86^{o}C in an Ultra deep freezer, then transferred to 100% acetone at -20^{o}C for a further 3 d. Samples left in 100% acetone at -86^{o}C (i.e. freeze substitution medium 3) for 3 d were transferred to fresh 100% acetone at -20^{o}C for the following 3 d. After dehydration at -20^{o}C, samples were left at room temperature to thaw, rinsed with phosphate buffer (pH 7.2 for x 5min), then post-fixed with 0.5% aqueous OsO_4 for 1 h. The acetone was replaced with increasing concentrations of ethanol at room temperature using the following method:

75% acetone + 25% ethanol (2 x 15 min)
50% acetone + 50% ethanol (2 x 15 min)
25% acetone + 75% ethanol (2 x 15 min)
100% ethanol (2 x 30 min)

To infiltrate the samples, the ethanol was replaced with increasing concentrations of LR white resin using the prescribed protocol and embedded in whole resin, then transferred to individual gelatine capsules (size 3) with plain LR white resin and polymerised. Semi thin sections were cut using the Reichert-Jung Ultracut E microtome and slides prepared as above. Little detail was visible at light level before ultra thin sections were cut. However, material showed significant damage.

2.2.4.5 Microwave radiation fixation

Cubes of root meristematic tissue, approximately 0.5 x 0.5 x 0.5 mm, was excised in citric acid, dabbed dry on filter paper to remove excess citric acid solution, and placed in a No. 1 pill vial (approximately 10 x 50 mm), containing 3 ml 2.5% glutaraldehyde incorporating 1% caffeine made in a phosphate buffer (pH 7.2). Control axes were fixed for 30 min in the glutaraldehyde solution followed by the standard TEM fixation protocol. Conventional procedures for dehydration in acetone, infiltration and embedding with Spurr's Resin were followed. For the first treatment, samples were fixed in the glutaraldehyde solution for 5 min followed by microwave fixation at maximum power in a standard household microwave (700 watts) for 10s. Four 200ml beakers, filled with cold tap water, were placed in each corner of the microwave to prevent the samples boiling or dehydrating. After 10 s, samples were removed and 5ml phosphate buffer (pH 7.2) cooled with LN was added immediately to stop heat fixation, followed by standard TEM processing. The second and third treatments were done in a similar manner, however fixation times in glutaraldehyde were

increased to 15 and 30 min respectively, before microwave fixation for 10 s. Each time samples were microwaved, the water in the beakers was replaced with cold tap water. Semi thin sections were cut using a Reichert-Jung Ultracut E microtome with a glass knife, picked up and slides were prepared as previously described. Ultrathin sections were cut using the same microtome and picked up on 600 mesh copper grids. Sections were double stained, first for 8min with uranyl acetate, rinsed with distilled water and thereafter with lead citrate (Reynolds, 1963) for 10 min followed by a second rinsing with distilled water. Sections were viewed using the Jeol JEM 1010 TEM at an accelerating voltage of 80kv. Images were captured Megaview 3 camera and analysed with the TEM imaging software, Analysis iTEM.

(Other fixation techniques, such as high pressure freezing and microwave fixation with an industrial microwave with variable power settings and a cold plate, were not assessed because of lack of availability of appropriate equipment.)

CHAPTER 3: RESULTS

3.1 Physiology

3.1.1 Storage in a saturated atmosphere

The water content (dry mass basis) of axes of *Syzygium cordatum* excised from seeds that had been exposed to various anti-fungal treatments and then stored either in a saturated atmosphere or at their original water content (w.c.) was measured at 2 weekly sampling intervals. Whole seeds were used to determine vigour and viability.

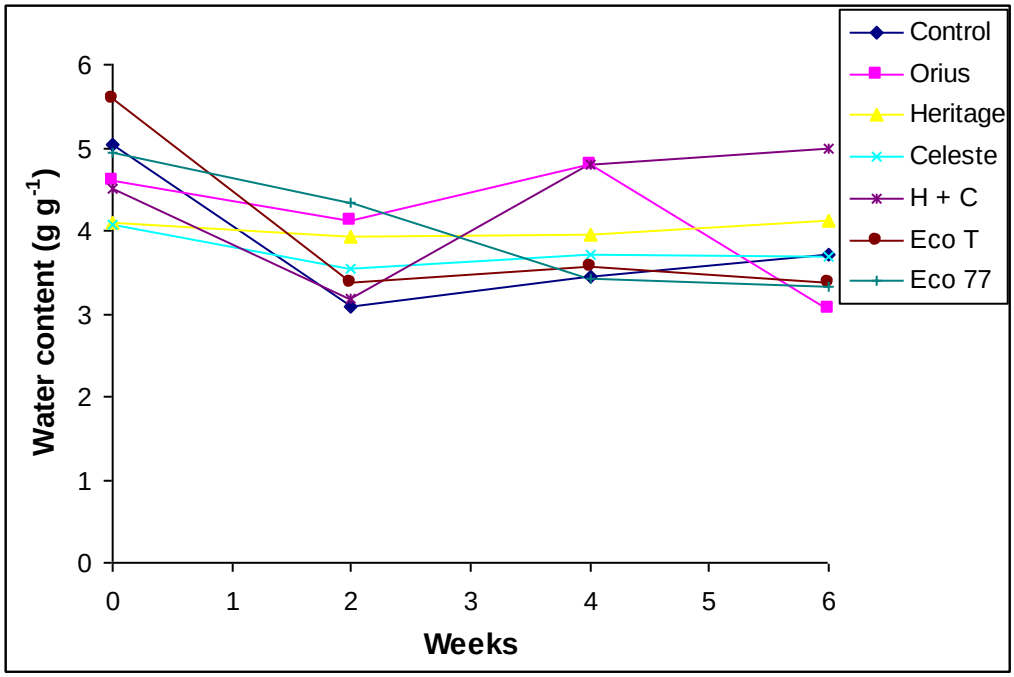


Figure 3.1: Mean water content of axes from seeds treated with the different fungicide treatments and stored in a saturated atmosphere (n=5)

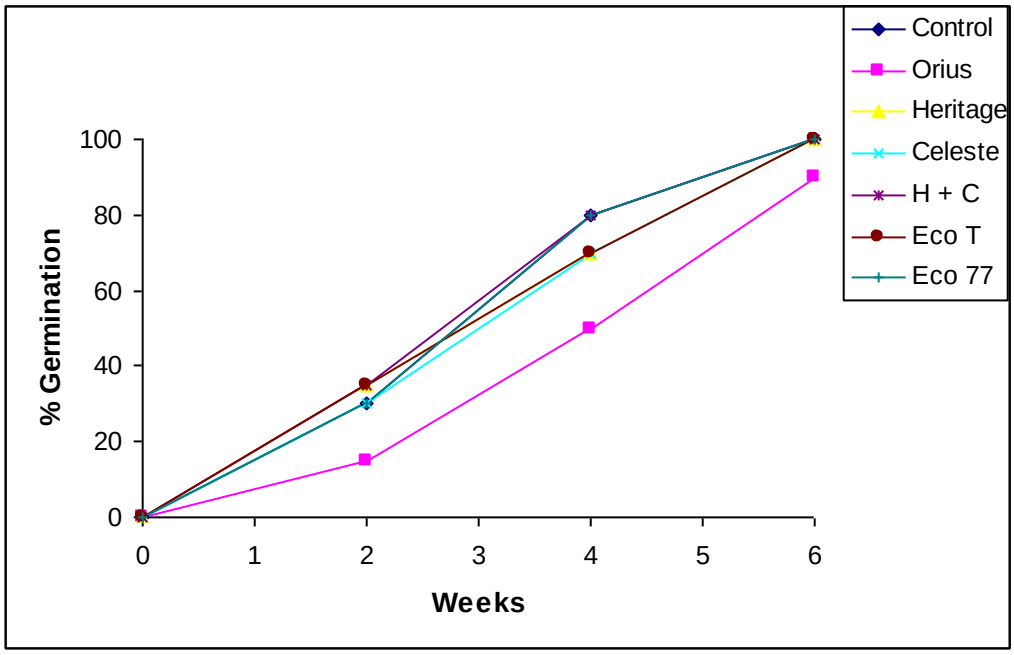


Figure 3.2: Percentage germination of seeds in storage (n≈150)

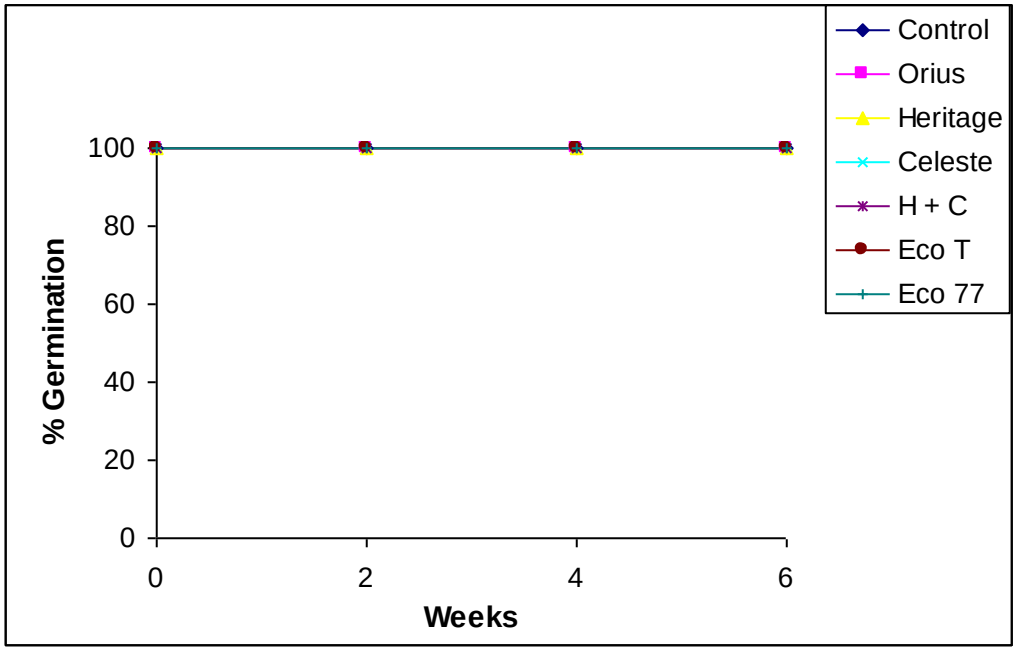


Figure 3.3: Percentage germination after *in vitro* germination (n=5)

Seeds from all treatments stored in a saturated atmosphere showed an initial decrease in axis w.c. with great variability with large SDs (Fig 3.1), however from 2-4 weeks in storage, axes from seeds from all treatments except Eco 77, showed a slight increase in water content. During weeks 4 – 6

water content of axes from seeds of the treatments control, Heritage and Celeste + Heritage increased, whereas the seeds from remaining treatments showed a slight decrease in axis w.c., while seeds treated with Orius showed the largest decrease in axis w.c. of 1.73 g g^{-1} (Fig 3.1). After 2 weeks (Fig 3.2), seeds treated with Heritage, Celeste + Heritage, and Eco T showed a 35% germination in storage; and seeds from the control, and Celeste and Eco 77 treatments showed a 30% germination in storage while seeds treated with Orius exhibited the least, at 15% germination. All treatments except Orius (90% germination) showed 100% germination in storage by week 6 which coincides with the greatest decrease of axis water content Vigour and viability remained constant throughout the saturated atmosphere storage trial as seeds from all sampling periods from all treatments showed signs of germination with the protrusion of a radicle 3 – 5 d after culturing onto water agar, and germination remained at 100% (Fig 3.3). All seeds showed signs of germination 3-4 d after plating and formed healthy seedlings after 14 d.

3.1.2 Storage at original seed water content

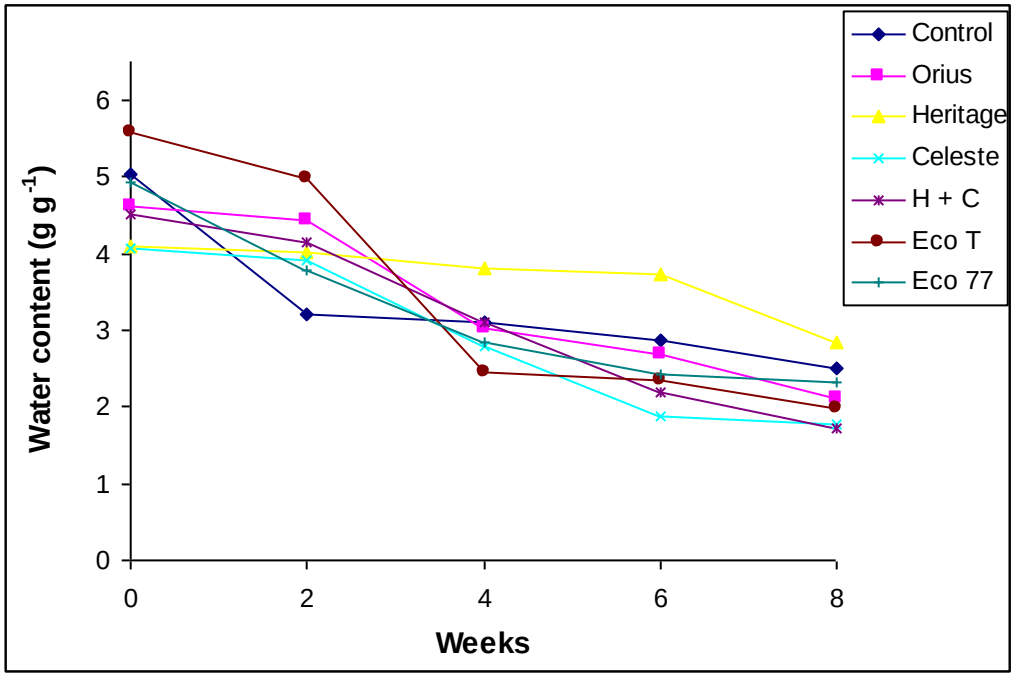


Figure 3.4: Mean water content of axes from seeds treated with the different fungicide treatments and stored at original seed water content (n=5)

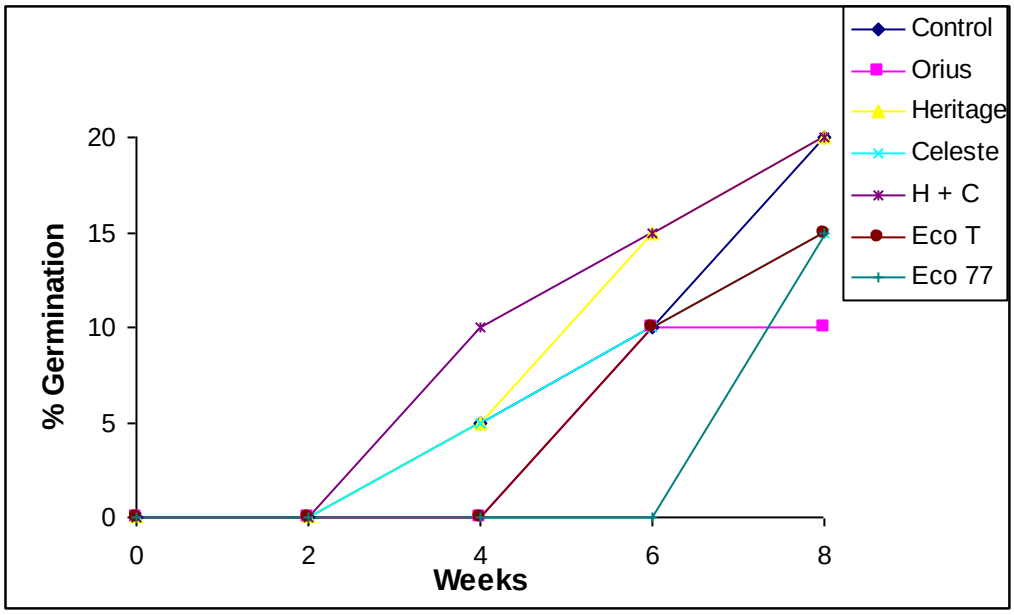


Figure 3.5: Percentage germination of seeds in storage (n≈150)

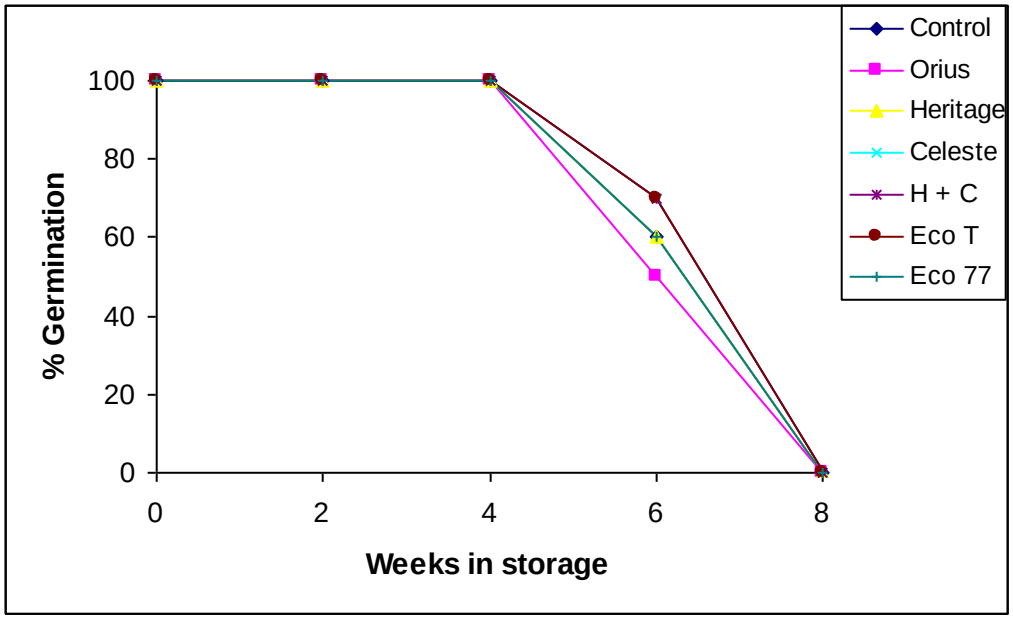


Figure 3.6: Percentage germination after *in vitro* germination (n=5)

Seeds from all treatments showed an overall decrease in w.c. during storage when stored at the original seed water content (Fig 3.4). The largest decrease in w.c. after 2 weeks was noted in seeds from the control which dropped from 5.0 g g^{-1} at week 0 to 3.2 g g^{-1} at week 2, after which, water content remained relatively constant at around 3.0 g g^{-1} from 4 weeks. After 4 weeks in storage,

seeds from the Control, and the treatments Heritage + Celeste and Celeste showed 5% germination in storage and seeds treated with Heritage showed 10% germination, while the remaining treatments showed no germination in storage (Fig 3.5). At this time, axes from seeds treated with Heritage also had the highest water content (3.8 g g^{-1}). By week 6, all treatments except Eco77 showed some germination in storage. By week 8, all treatments showed some germination in storage; however, seeds treated with Orius showed the lowest yield at 10% germination in storage. After week 6, all treatments showed a decrease in germination after whole seeds were plated onto water agar, and seeds treated with Orius showing the greatest decrease (50%) in germination. *In vitro* germination continued to decrease with time, and by week 8, only seeds treated with Heritage showed any germination (10%) after plating, while the remaining treatments were no longer viable (Fig 3.6). The seeds treated with Heritage also showed the highest axis water content, 2.8 $g.g^{-1}$, after 8 weeks in storage.

Table 3.1: Table showing the significant differences between storage regimes, storage fungicide treatments and storage time at 2 weekly intervals after a univariant ANOVA

	Significance (p value)		
	2 weeks	4 weeks	6 weeks
Storage	0.033	0.000	0.000
Treatments	0.004	0.000	0.001
Storage*Treatments	0.378	0.004	0.002

At two-weekly intervals, stored seeds were sampled and the water content for the storage regime (i.e. saturated atmosphere and original water content) and fungicide treatment was determined. A univariant anova with two fixed factors, storage regime and fungicide treatment, was carried out at each sampling period and the significance of differences between storage regime and original values, and between fungicide treatment and original value were ascertained (Table 3.1). Week 0 and 8 were not compared as the results for week 0 were used for both storage regimes and by week 8, all seeds stored in the saturated atmosphere had germinated, so no comparison could be made. After 2, 4 and 6 weeks in storage, there were significant differences in the interaction between storage regime and the different fungicide treatments. However significant interactions were not noted between regime and treatments after 2 weeks. After 4 and 6 weeks, there were significant interaction, between storage regime and the different fungicide treatments.

3.1.3 Flash drying of excised axes

Excised axes of *S. cordatum* were flash dried and water content and viability were measured.

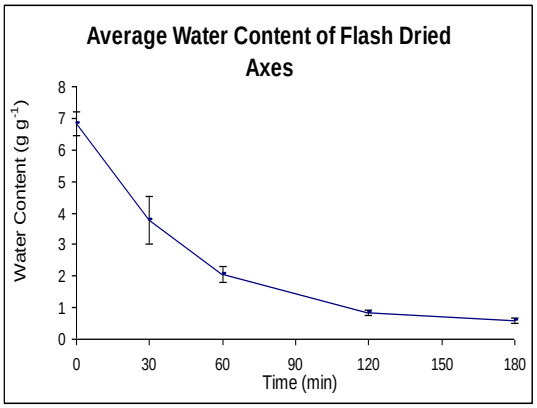


Figure 3.7: Mean water contents of flash dried axes with time after flash drying (n=10). Error bars in this and subsequent Figures represent ± one standard deviation.

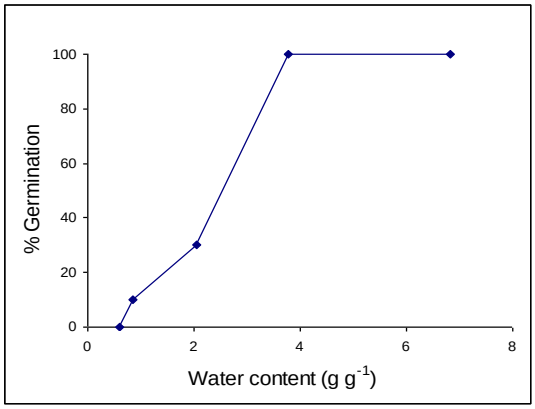


Figure 3.8: Percentage germination of flash dried axes as a function of w.c. (n=20)

After 30 min of flash drying, the water content of axes decreased from 6.8 to 3.7 g g^{-1} (Fig 3.7). This initial decrease in w.c. did not appear to have an effect on viability as *in vitro* germination on full strength MS culture medium remained at 100% (Fig 3.8). However, after 60 min flash drying, axis w.c. decreased by 1.7 g g^{-1} to 2.0 g g^{-1} (Fig 3.7), and *in vitro* germination dropped to 30% (Fig 3.8). Axis w.c. loss after 60 min of flash drying was more gradual. After 120 min flash drying, axis water content was reduced to 0.85 g g^{-1} and germination was only 10%. Following 180 min flash drying, axis w.c. had reached 0.5 g g^{-1} and no axes were viable.

3.1.4 Slow drying of whole seeds over silica gel

Whole seeds of *S. cordatum* were weighed, dehydrated over silica gel then re-weighed, and the percentage water lost calculated, until the desired level of water loss was achieved. This was carried out to determine the effects of slow drying on seeds, as well as the water content at which axes lose viability. The axes were excised and the water content determined on a dry mass basis. Viability was tested by culturing the flash dried axes onto full strength MS medium.

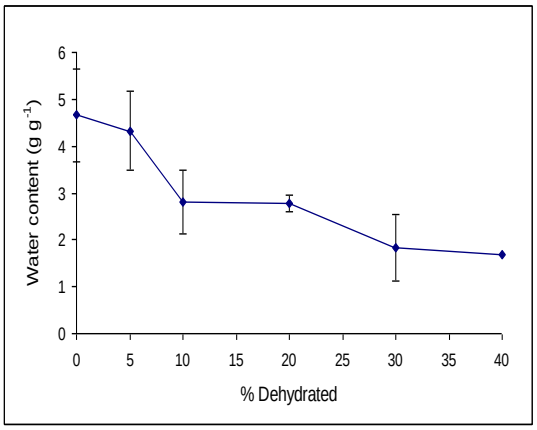


Figure 3.9: Water content of axes plotted dehydration after dehydration of whole seeds over silica gel (n=10)

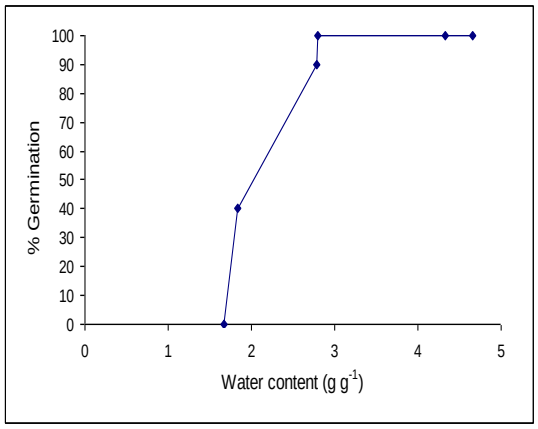
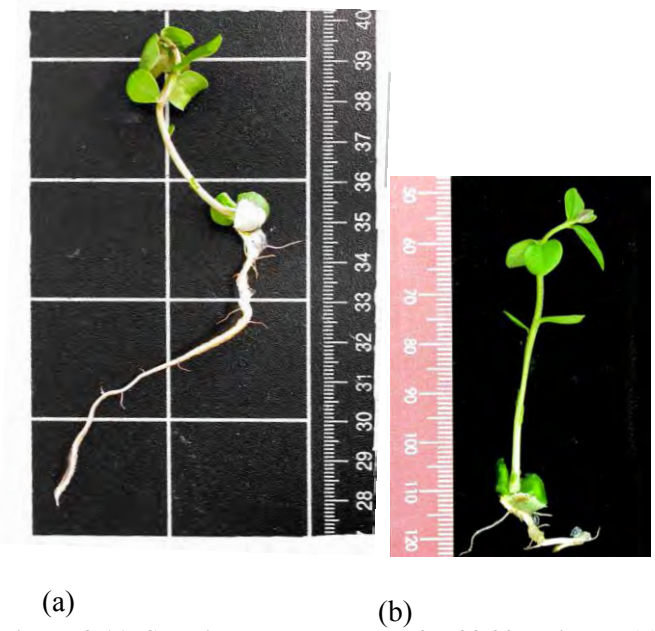


Figure 3.10: Percentage germination of axes from seeds dehydrated over silica gel plotted as a function of w.c. (n=10)

Axial w.c. showed a steady decline (Fig 3.9), with a decrease of 2.1 g g^{-1} from 4.7 to 2.8 g g^{-1}; however, viability was unaffected and remained at 100% after *in vitro* germination (Fig 3.10) on full strength MS medium. At 2.78 g g^{-1} (20% dehydration) axis w.c., viability dropped to 90%. At 1.83 g g^{-1} (30% dehydration) axis w.c., viability dropped to 40% after *in vitro* germination. However at 1.6 g g^{-1} (40% dehydration), the decrease in w.c. was only 0.16 g g^{-1} more than the w.c. at 30%, and axes were no longer viable.



(a) (b)

Figure 3.11: Seedlings that have lost 0 – 20 % moisture (a) and 30 % moisture (b)

Approximately 41 d after culturing onto water agar, seeds losing up to 20 % moisture (Fig: 3.11a) produced normal seedlings, while seeds losing 30% moisture (Fig: 3.11b) produced seedlings with poor root formation. Roots appeared stunted and thicker than normal.

3.1.5 Hydration of whole seeds

Whole seeds were weighed and soaked in distilled water for specific periods, after which they were removed, the axes excised and the axis water content determined on a dry mass basis. The aim of this experiment was to determine the necessary amount of time required for the soaking seeds to absorb the fungicides used in the storage treatment.

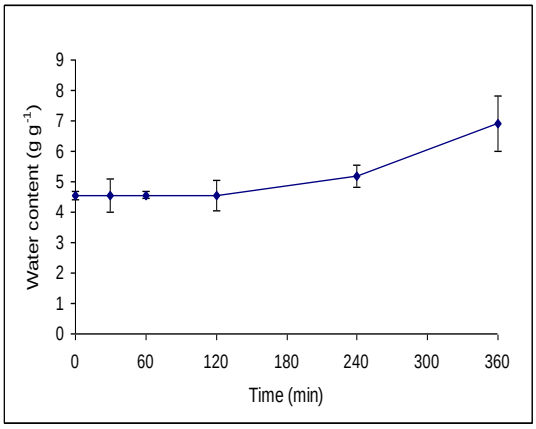


Figure 3.12: Water content of axes after hydration of whole seeds in distilled water as a function of time after hydration (n=10)

There was no increase in axis w.c. over the first 120 min (Fig 3.12). From 120 – 240 min w.c. increased from 4.56 to 5.18 g g^{-1} respectively, and from 240 – 360 min water uptake was more rapid increasing to 6.92 g g^{-1}. Viability remained unchanged at 100% for each soaking time.

3.2 Tissue Culture

3.2.1 Somatic embryogenesis/callus production

Flash dried axes lost considerable viability on drying to a w.c. of ≈ 2.0 g g^{-1}, which is far too high for cryopreservation (w.c. ≈ 0.3 – 0.4 g g^{-1}), hence a suitable alternate explant was sought for cryopreservation, and attempts to generate somatic embryos were made using various explants.

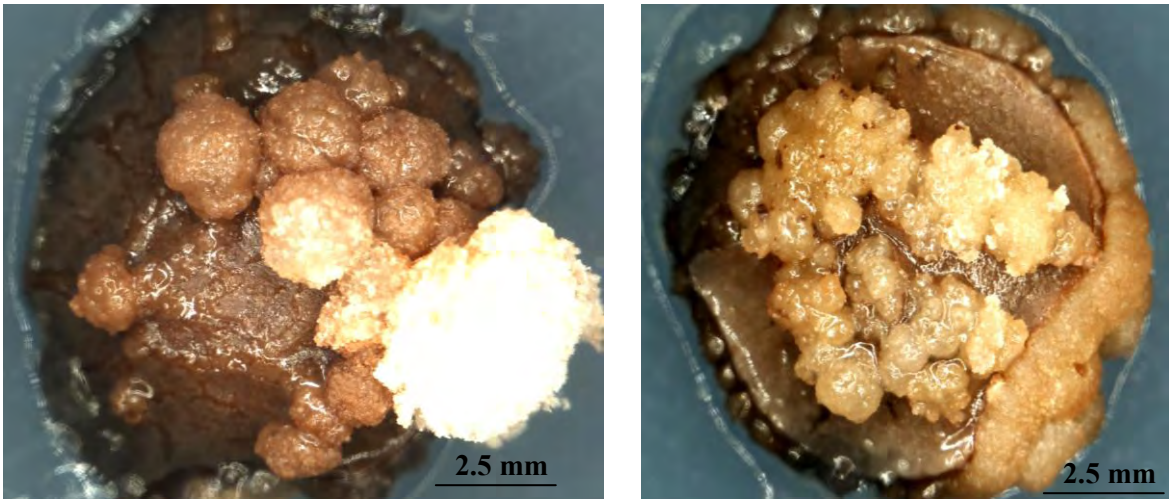
Prepared cotyledons were cultured onto media, poured into Petri plates promoting somatic embryogenesis and the results were recorded. Media differed in the concentration of MS and the addition of various plant growth regulators.

Stereomicroscope images of callus produced

Cotyledonary explants on Treatment 1 (Table: 2.4) produced swollen cotyledons that produced slightly green nodules on 50% of cotyledons cultured onto the medium (Fig: 3.13). The remaining 50% of cultures were either lost to contamination or were considered dead after 14 d when no result was noted and cotyledonary segments appeared black. (n=30)

Figure 3.13: Treatment 1. Cotyledonary explant on full strength MS, 0.5 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose

After 12 weeks in culture, Treatment 2 produced a brown nodular callus on 50% of explants, approximately 1 - 2 mm in length (Fig: 3.14b). A white crystalline callus was also observed on 7% of cultures (Fig 3.14a). (n=30)



(a) (b)

Figure 3.14: Treatment 2. Cotyledonary explant cultured onto medium containing full strength MS, 1 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose

After 12 weeks in culture, 50% of cotyledonary explants in Treatment 3 produced a light brown callus without organised structures (Fig: 3.15). A white powdery callus was also present on 10% of cultures. The remaining 50% of cultures were lost to contamination or considered dead when cotyledonary segment, appeared black. (n=30).

Figure 3.15: Treatment 3. Cotyledonary explant cultured onto medium containing full strength MS, 1 mg l^{-1} 2,4-D and 103 g l^{-1} sucrose

After 10 weeks in culture, the surface of 90% of explants on Treatment 4 peeled off, exposing a green under surface that produced a nodular callus 8 weeks later (Fig: 3.16). The remaining 10 % of explants were considered dead. (n=30)

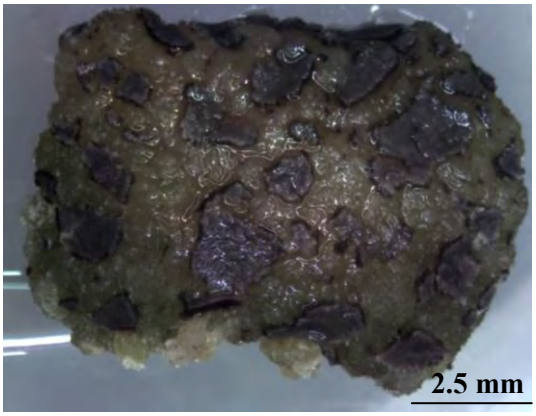


Figure 3.16: Treatment 4. Cotyledonary explant cultured onto medium containing ½ strength MS, 0.1 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose

Cotyledonary segments produced a crystalline white callus on 30% of cultures (Fig 3.17) on Treatment 5 in 5 weeks. During sub-culturing, callus broke off in powdery segments. Sub-culturing onto shoot and root promoting media produced no further results. Callus appeared dead after 10 d. (n=30)

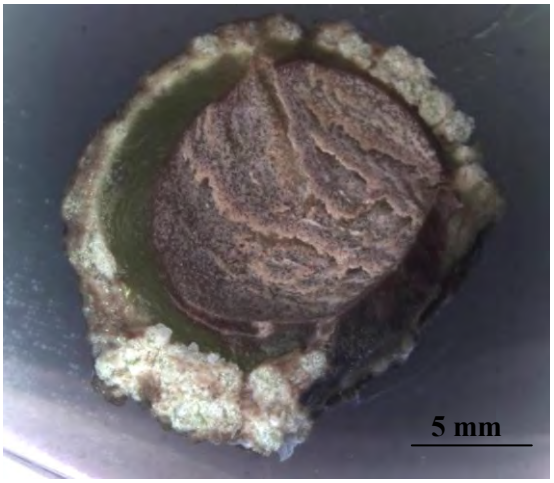


Figure 3.17: Treatment 5. Cotyledonary explant cultured onto medium containing full strength MS, 2 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose

After 12 weeks in culture, cotyledonary explants in Treatment 6 produced brown (Fig: 3.18) and Treatments 7 produced white crystalline (Fig: 3.19) callus on approximately 53% of cultures (n=30).

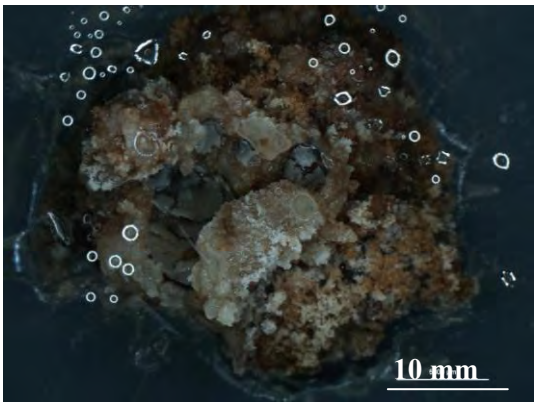


Figure 3.18: Treatment 6. Cotyledonary explant cultured onto medium containing full strength MS, 3 mg l^{-1} 2,4-D and 30 g l^{-1} sucrose

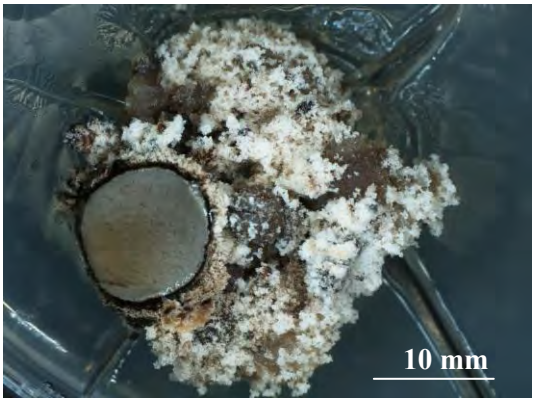


Figure 3.19: Treatment 7. Cotyledonary explant cultured onto medium containing full strength MS, 1 mg l^{-1} 2,4-D, 0.01 mg l^{-1} BAP and 30 g l^{-1} sucrose

After 12 weeks on Treatment 8, the callus produced on 50% of cotyledonary explants was green and powdery (Fig 3.20). After sub-culturing onto root and shoot promoting media, no further results were noted, and callus appeared dead after 10 d. The remaining 50% of explants that produced no result turned dark brown after 2 weeks and were considered dead. (n=30)

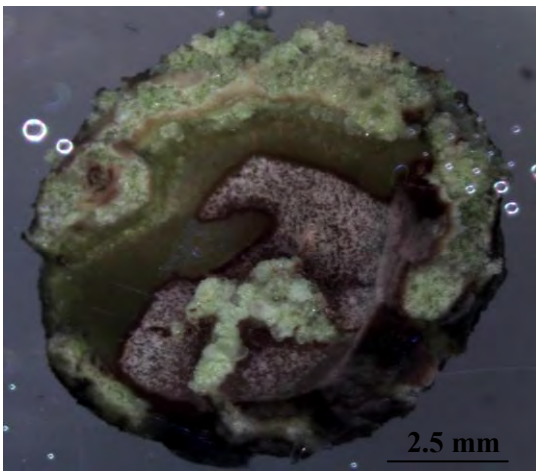


Figure 3.20: Treatment 8. Cotyledonary explant cultured onto medium containing full strength MS, 1 mg l^{-1} 2,4-D, 0.1 mg l^{-1} BAP and 30 g l^{-1} sucrose

A white, slightly translucent nodular callus (Fig 3.21) was produced on 40% of cultures of Treatment 9 after 12 weeks. The remaining 60% was considered dead. Axes cultured onto the same medium germinated after 10 d (n=30).

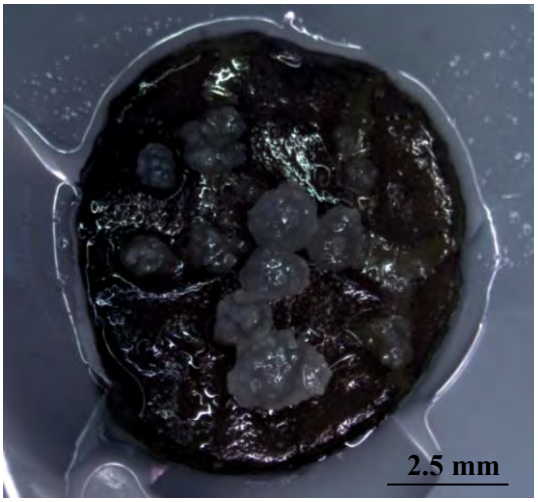


Figure 3.21: Treatment 9. Cotyledonary explant cultured onto medium containing full strength MS, 1 mg l^{-1} 2,4-D, 0.5 mg l^{-1} BAP and 30 g l^{-1} sucrose

Light microscopy of calli produced by explants cultured onto somatic embryogenesis media

All nodular calluses from somatic embryogenesis (Treatments 1-9) showed that similar cell types were present (Fig 3.22). Cells were large, circular and appeared to have little cellular detail with thin cell walls.

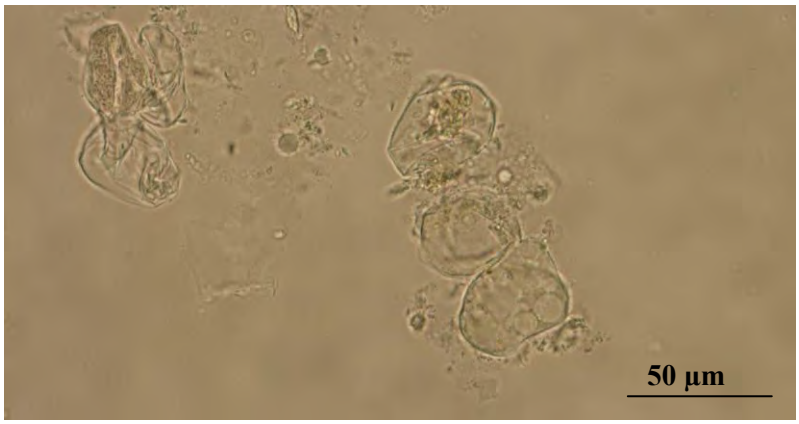


Figure 3.22: Light micrograph of nodular callus from Treatments 1-9 showed similar cells

Samples taken from crystalline callus tissue showed cells that were oblong or rod like and again appeared to have little cellular detail (Fig 3.23). However cells possessed cell walls.

Figure 3.23: Light micrograph of crystalline callus from Treatments 1-9 showed similar cells

3.2.2. Organogenesis/alternate explants

3.2.2.1 Shoot production

Leaves as explants

Leaves taken from trees from which the fruit were harvested were cultured on shoot inducing media Treatments 1 (full strength MS and 0.75 mg l^{-1} BAP) and 2 (½ strength MS and 0.75 mg l^{-1} BAP) produced no result as contamination occurred within 2 d of culturing. Sterilisation time was increased to 8 and then 10 min. However, this only promoted tissue death. Leaves from *in vitro* plantlets that were grown to test the viability of stored seeds during the storage trail were also used. However the cut edges of leaves turned brown and curled upwards, therefore treatments using leaves were not pursued.

Nodules as explants

Nodules produced by cotyledonary explants cultured onto somatic embryogenesis media (Treatments 2, 4 and 9) (Fig: 3.24) were excised and sub-cultured onto shoot inducing media Treatment 1 (full strength MS and 0.75 mg l^{-1} BAP), and 3(full strength MS, 1 mg l^{-1} BAP and 0.5 mg l^{-1}), in an attempt to stimulate the nodules to produce shoots which would later be induced to produce roots. However, neither treatment showed any further result. Approximately 8 - 10 d after sub-culturing, nodules were considered dead as they had turned dark brown and appeared shrunken.

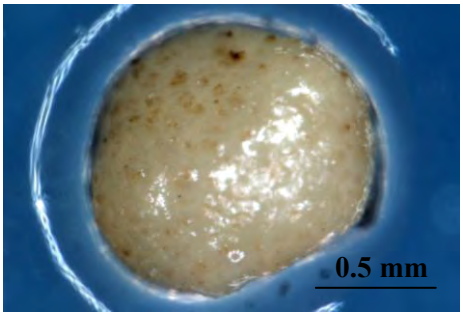
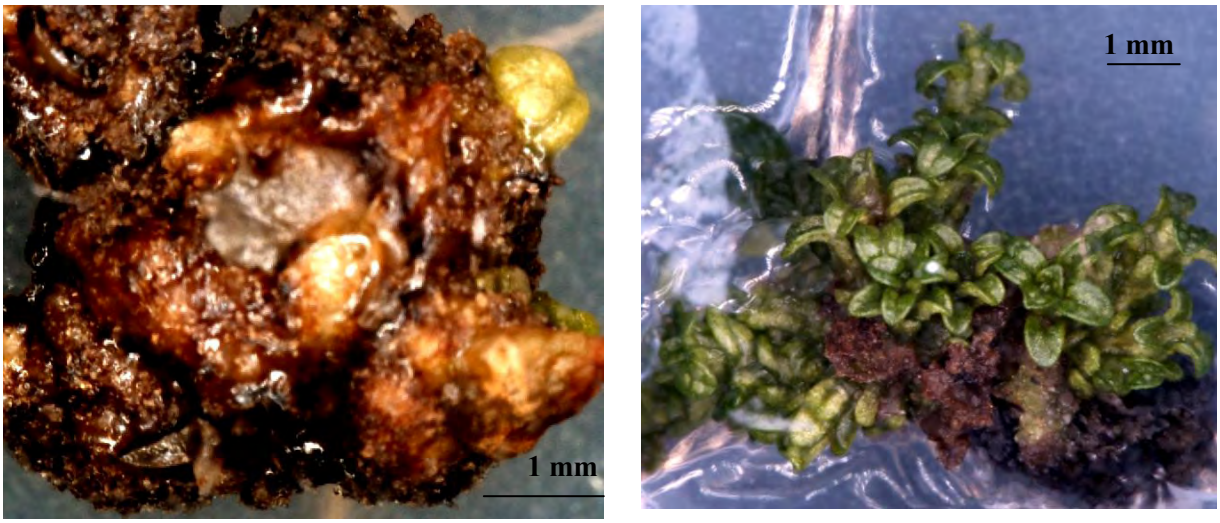


Figure 3.24: Nodular callus produced by cotyledonary explants on somatic embryogenesis media were excised and sub-cultured onto media promoting shoot development

Zygotic axes as explants

Zygotic axes cultured onto Treatment 3 (full strength MS, 1 mg l^{-1} BAP and 0.5 mg l^{-1} NAA) produced callus after 5 weeks (Fig: 3.25a), and sub-culturing onto the same medium produced 8 -14 adventitious shoots 10 – 12 weeks later (Fig: 3.25b).



(a) (b)

Figure 3.25: Zygotic axes used to produce microshoots

Shoot production was sometimes asynchronous as a few explants produced early shoots which grew to ≈ 20 mm long while shoots produced later remained small (≈ 2-3 mm) (Fig: 3.26). A medium promoting shoot elongation was then tested to increase the rate at which 2 mm shoots grew to ≈ 20 mm long.

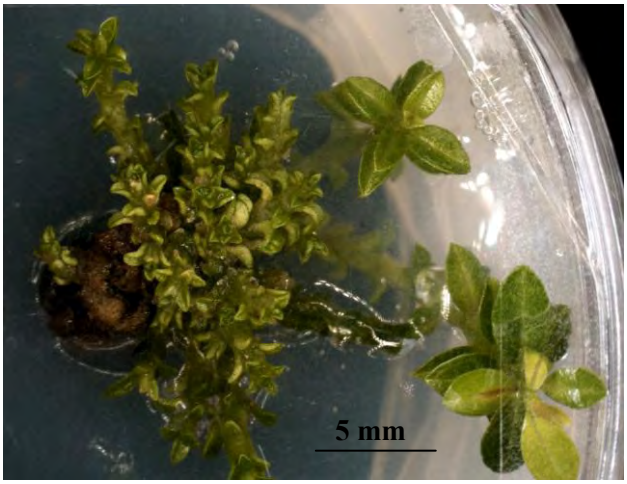


Figure 3.26: Asynchronous shoots produced by zygotic axes

3.2.2.2 Shoot elongation

Shoot elongation Treatments 1 (full strength MS, 1 mg l^{-1} BAP, 0.5 mg l^{-1} NAA and 30 g l^{-1} sucrose), 2 (1/2 strength MS and 30 g l^{-1} sucrose) and 3 (full strength MS and 30 g l^{-1} sucrose) promoted shoot elongation within 4 weeks when whole explants of adventitious shoots were sub-cultured in Petri dishes. Whole explants sub-cultured into 100 ml culture bottles died within 6 d of culturing (Fig 3.27), presumably due to the increased air environment which may have lead to drying of the tissue.

Figure 3.27: Explant with asynchronous shoots sub-cultured into 100 ml culture bottle turned necrotic

3.2.2.3 Root production

Rooting Treatments 1 (full strength MS, 3 mg l^{-1} NAA and 30 g l^{-1} sucrose) and 2 (full strength MS, 0.5 mg l^{-1} BAP, 1 mg l^{-1} NAA and 30 g l^{-1} sucrose) produced no result when excised shoots and whole explants of multiple adventitious shoots were sub-cultured onto them. Whole explants sub-cultured onto Treatment 3 (full strength MS, 0.1 mg l^{-1} and 30 g l^{-1} sucrose) produced adventitious roots 12 weeks after sub-culturing (Fig: 3.28). Excised shoots sub-cultured onto Treatment 4 (full strength MS, 0.2 mg l^{-1} GA_3 and 30 g l^{-1} sucrose) produced roots within 7 weeks (Fig: 3.29) and produced no roots when sub-cultured onto Treatment 5 (full strength MS, 0.5 mg l^{-1} GA_3 and 30 g l^{-1} sucrose).

Figure 3.28: Treatment 3. Whole explants on media containing full strength MS, 0.1 mg l^{-1} GA_3 and 30 g l^{-1} sucrose

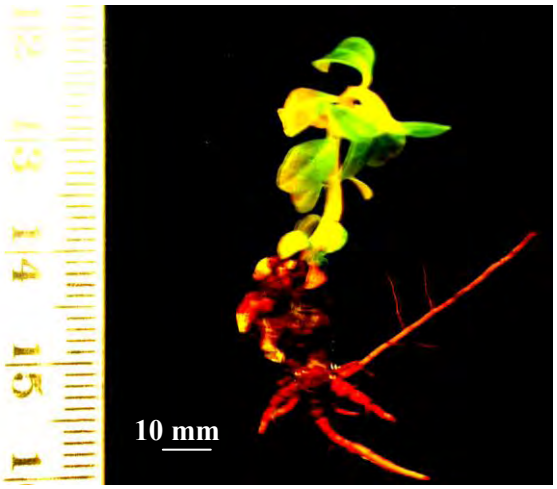


Figure 3.29: Treatment 4. Excised shoots on media containing full strength MS, 0.2 mg l^{-1} GA_3 and 30 g l^{-1} sucrose

Zygotic axes were unsuitable for cryopreservation as they were too sensitive to desiccation. Alternative explants were used to produce somatic embryos. However, as this was unsuccessful, further investigations for cryopreservation were not pursued. Indirect organogenesis was used to produce adventitious shoots that were induced to produce roots.

3.3 Transmission electron microscopy

3.3.1 Glutaraldehyde fixation

At the light-level, cells fixed with glutaraldehyde for 24 h using the conventional TEM method of fixation appeared badly damaged and fragmented (Fig: 3.30). Large holes in tissue did not allow for ultrathin sections to be cut. Tissue was brittle and tore easily. Little detail was viewable as cellular damage was severe.

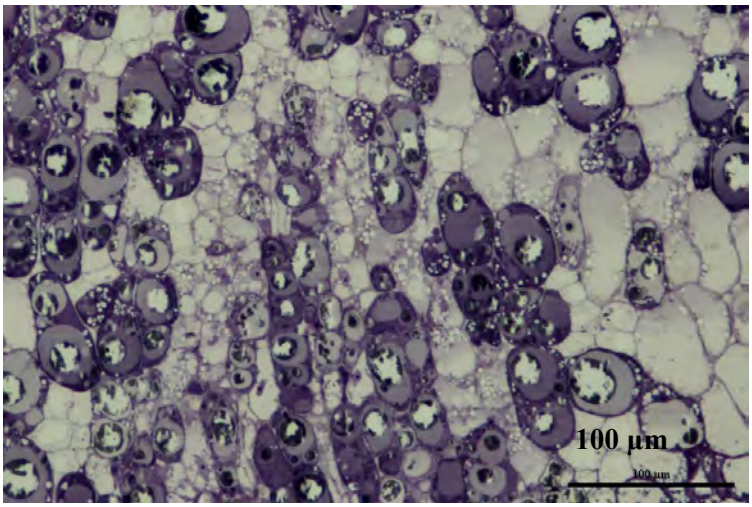


Figure 3.30: Root meristematic tissue fixed using 2.5% glutaraldehyde

3.3.2 Permanganate fixation

Results did not improve with permanganate fixation (Fig: 3.31). Preparation at both 0 and 4^{o}C and at all fixation times produced similar results. Tissue was brittle and tore when cutting semi thin sections. Attempts at cutting ultrathin sections produced fragmented tissue that disintegrated when in contact with water.

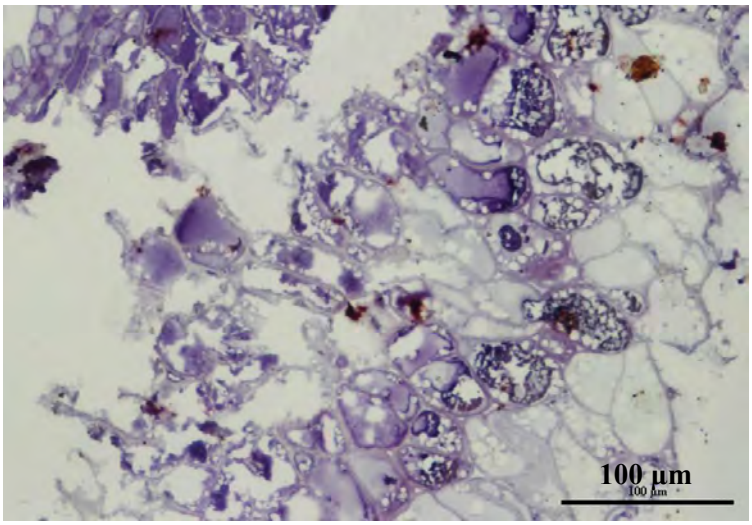


Figure 3.31: Root meristematic tissue fixed using 1% $KMnO_4$ at 0^oC then processed in the conventional TEM manner

3.3.3 Osmium fixation

Fixation with OsO_4 allowed for only surface layers of cells to be fixed (Fig: 3.32). The brown area (A), which is the meristematic region and the area of interest, remained un-fixed. During semi thin sectioning, poorly fixed and infiltrated areas broke off into fragments and did not allow for ultrathin sections to be cut.

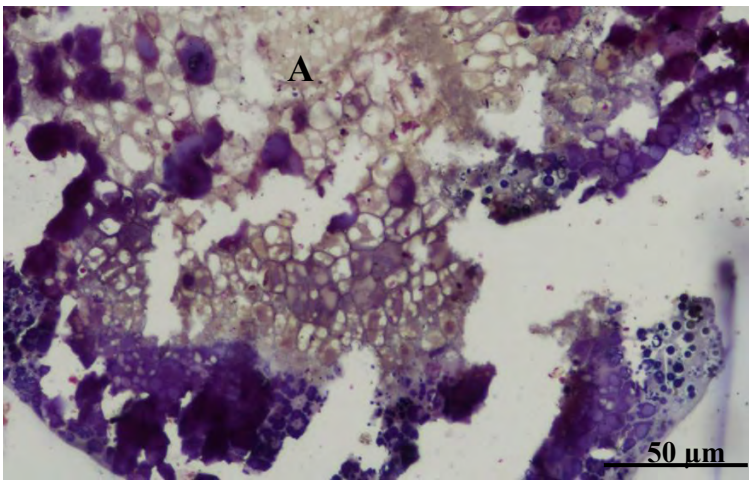


Figure 3.32: Root meristematic tissue fixed using 1% OsO_4

3.3.4 Freeze substitution

All 3 cryo treatments with freeze substitution showed similar results with fine (Fig: 3.33a) and large (Fig: 3.33b) ice crystal damage, and little cellular detail was visible. Cell walls and organelles were indistinguishable.

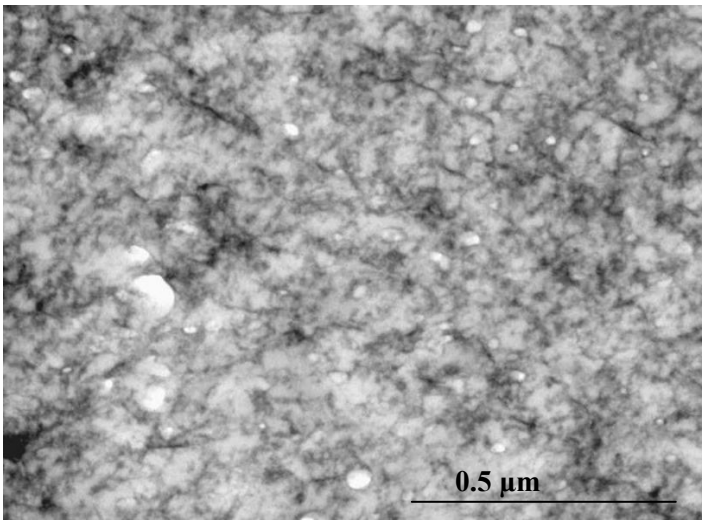


Figure 3.33a: Root meristematic tissue cryo-processed with plunge-freeze and freeze-substitution

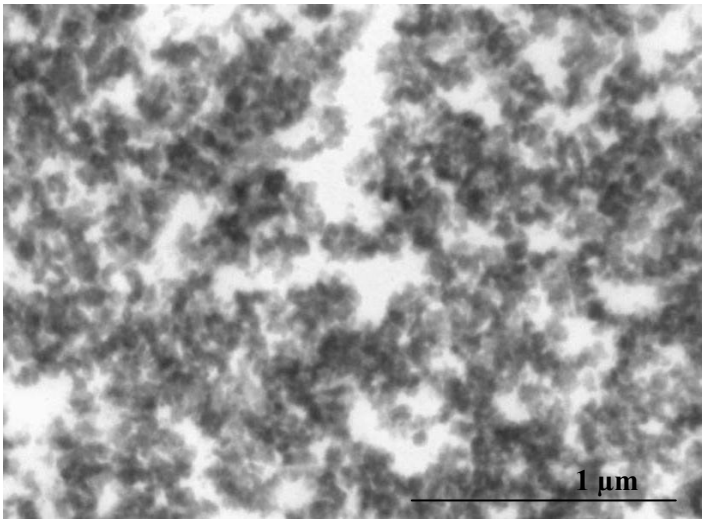


Figure 3.33b: Root meristematic tissue cryo-processed with plunge-freeze and freeze-substitution

3.3.5 Heat radiation fixation

3.3.5.1 Control material

The control material was fixed for 30 min with glutaraldehyde only, without heat radiation fixation. Fixation was poor and semi-thin sections cut were brittle while ultrathin sections were not obtainable, therefore not viewable using the TEM.

3.3.5.2 Five minute fixation

When the tissue was fixed with glutaraldehyde for 5 min, followed by microwave-fixation for 10 s, section quality was not ideal (Fig: 3.34), but semi-thin sections were obtainable. Sections showed some degree of tearing. Cell walls (CW) were distinct and membranes were visible, giving poor detail to membrane bound organelles.

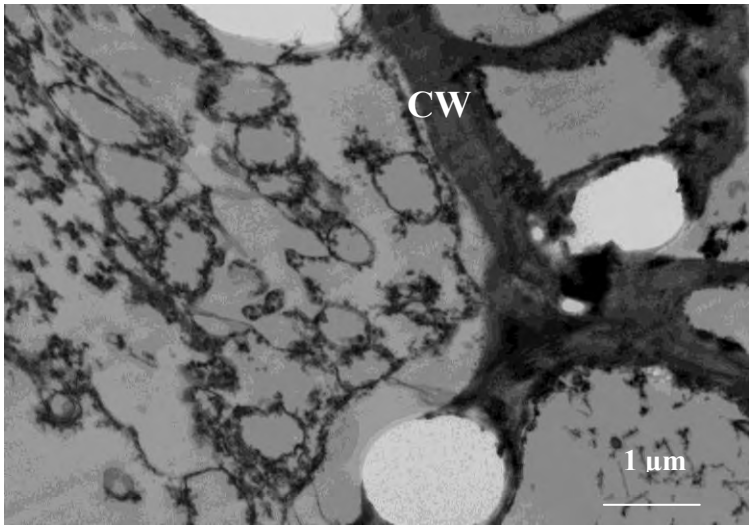


Figure 3.34: Root meristematic tissue fixed for 5 min with glutaraldehyde, then heat fixed for 10 s

3.3.5.3 Fifteen minute fixation

When material was fixed with glutaraldehyde for 15 min, and thereafter microwave-fixed for 10 s, section quality was greatly improved. There was little or no tearing (Fig: 3.35). Cell walls (CW) and membrane bound organelles were clearly visible e.g. Golgi body (A) found in the cell.

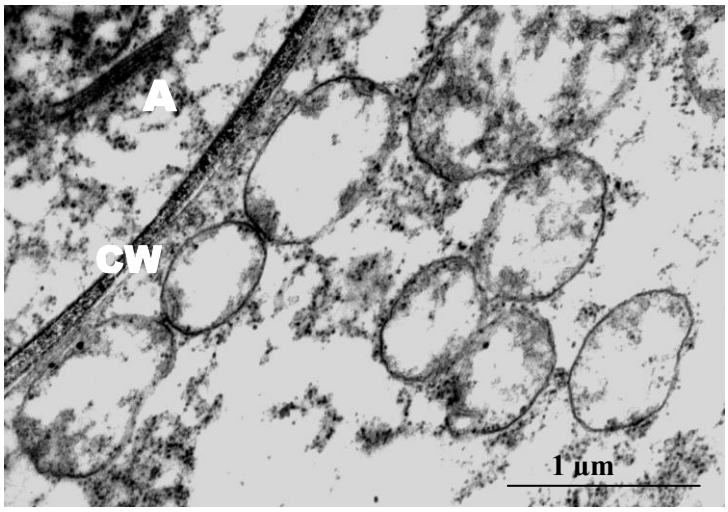


Figure 3.35: Root meristematic tissue fixed with glutaraldehyde for 15 min then, heat fixed for 10 s

3.3.5.4 Thirty min fixation

Section quality showed a noticeable improvement over the conventional method of TEM fixation, when samples were microwave-fixed for 30 min, then microwave fixed for 10 s (Fig: 3.36). Ultrathin sections were now obtainable and cell walls (CW) and organellar structures, such as mitochondria (M) were clearly visible. However large bodies of phenolics were present and would tear from the section (A), with detrimental effect on section quality and stability under the electron beam.

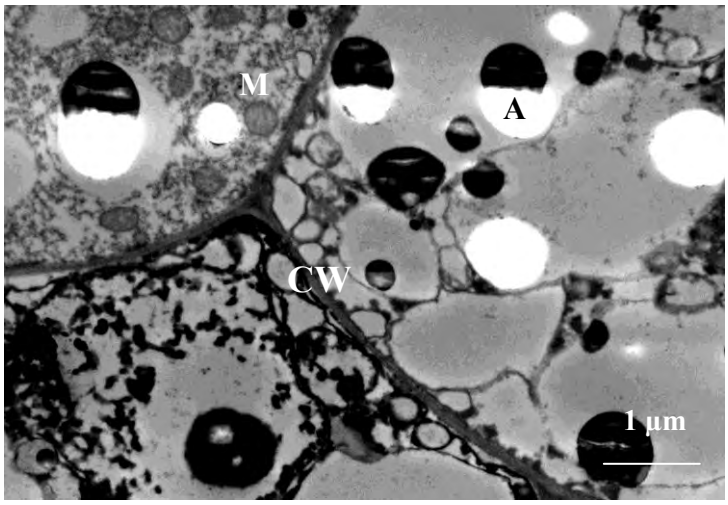


Figure 3.36: Root meristematic tissue fixed with glutaraldehyde for 30 min then, heat fixed for 10 s

CHAPTER 4: DISCUSSION

The initial aim of this project was to determine the potential for long-term storage of *Syzygium cordatum* germplasm. In order to achieve this, the recalcitrant nature of the seeds needed to be determined through various physiological studies. Once the recalcitrant nature of the seeds was determined, methods of miropropagation using various explants were tested and the potential for cryopreservation assessed. Additionally, optimum methods of TEM fixation were developed to enhance studies of this material.

4.1 Physiology

4.1.1 Drying rate and storage

Whole seeds of *S. cordatum* dried slowly in bags of silica gel to an axis water of 1.8 g g^{-1} showed a 40% viability (Fig 3.10) and when this water content was further reduced to 1.6 g g^{-1}, the seeds were no longer viable. However, axes of *S. cordatum* dried rapidly to water contents of 0.85 g g^{-1} (Fig 3.7) showed a 10% seed viability (Fig 3.8). This suggests that the seeds are desiccation sensitive, and metabolism derived damage accumulates when seeds are slowly dried. However, rapid drying does not allow for sufficient time for this damage to accumulate. The fact that the response to slow drying was indicative of the accumulation of damage also suggests that metabolism is ongoing, which indicates that the seeds are recalcitrant and that the w.c. at which all viability was lost via rapid drying was 0.61 g g^{-1}. However, it must be noted that variation in the minimum water content at which desiccation damage is induced, can occur between species and individual seeds of the same species (Chin and Roberts, 1980). Also, different moisture contents of freshly harvested seeds of a single cultivar may contribute to this variation (Chin and Roberts, 1980).

In vitro germination of *S. cordatum* was higher for seeds stored in a saturated atmosphere (Fig 3.3) when compared with seeds stored at original water content (Fig 3.6), indicating that the seeds are desiccation sensitive, as seeds stored at original water content did also lose water during storage and had a lower water content (Fig 3.4) than seeds stored in a saturated atmosphere (Fig: 3.1). The slow rate at which water was lost also contributed to the loss of viability of the seeds stored at the original water content.

4.1.2 Chilling damage

Unpublished data indicate that seeds treated with the fungicides and stored at 25^{o}C for both regimes and were subjected to rapidly proliferation of fungi. After 2 d, the seeds were unusable. Seeds treated with the fungicide treatments and stored at 4-6^{o}C for both regimes were no longer viable after 7 d of storage, indicating that they are chilling sensitive to temperatures of 4-6^{o}C. Seeds stored at a temperature of 16^{o}C retained 100% viability after 6 weeks in storage when stored in a saturated atmosphere (Fig: 3.3) and 50 – 70% viability (depending on fungicide treatment) (Fig: 3.6), indicating that they are not chilling sensitive to temperatures of 16^{o}C.

4.1.3 Germination

Seeds of *S. cordatum* stored at 16^{o}C remained viable for 6 weeks when stored in a saturated atmosphere (Fig 3.1) and 8 weeks when storage at original seed water content (Fig 3.4). The seeds stored in a saturated atmosphere were at a higher water content and showed a 100% germination in storage across all treatments by week 6 (Fig 3.2). By week 8, seeds stored at the original water content showed a 100% germination in storage (Fig 3.5), which is a characteristic of recalcitrant seeds, thereby confirming that seeds of *S. cordatum* display recalcitrant features. Also seeds for both storage regimes (i.e at original w.c and at saturated atmosphere) were initially dried in a laminar flow unit for 10 min which may have reduced the water content to levels that were sufficiently high enough to prevent desiccation damage, yet low enough to prevent 100% germination in storage until week 6. Also, although the pericarp of ripe seeds was removed, the seed coat was left intact, which could have prevented earlier germination of the seeds in storage.

4.1.4 Microbial growth

In the present study, microbial contamination in storage was negligible with only the controls showing approximately 10% contamination by week 6 in both storage regimes. Application of the topical fungicides for 6 h would appear to have allowed sufficient time for the seeds to absorbsufficient bactericide and fungicide required (Fig 3.12). The procedure of initial surface sterilisation of the seeds by immersion in a 1% calcium hypochlorite solution, followed by treatment with the fungicides allowed for the proper mode of action by the fungicides. The fungicides, which are primarily protective or curative in the early stages of fungal proliferation, were not used to eradicate mature spore-producing fungi as that stage would have been killed by the

1% calcium hypochlorite solution. Seeds treated with triazole (Orius) showed a least germination in storage in a saturated atmosphere (Fig 3.2), or at the original water content (Fig 3.5), however post-storage *in vitro* germination remained high which suggests that a triazole based fungicide is a suitable treatment for seeds of *S. cordatum*.

4.2 Tissue culture

Skoog (1944) discovered that adding auxin to the medium stimulated the production of roots while inhibiting shoot formation (Dodds *et al.*, 1985). Shoot initiation in plant tissue culture requires a delicate balance between auxins and cytokinins in the medium. Excised embryonic axes of *S. cordatum* used as explants in shoot initiation indirectly produced microshoots. After 5 weeks in culture, a mass of callus was produced (Fig 3.25a), and 10 - 12 weeks later after continual sub-culturing onto the same medium microshoots were produced (Fig 3.25b). The asynchronous production of shoots resulted in varying developmental stages of the shoots. Several shoots had elongated while those produced at a later stage were much smaller (Fig 3.26). Sub-culturing onto the same medium or onto PGR free medium allowed the microshoots to elongate to a sufficient length for excision and transfer to media containing higher levels of the synthetic cytokinin BAP and lower levels of the synthetic auxin NAA, promoting root production. However, explants with microshoots sub-cultured into 100 ml culture bottles died (Fig 3.27) whereas explants sub-cultured into Petri dishes (Fig 3.25b) elongated. This could be due to the extra air space in the culture bottle permitted drying of the tissue. According to Hartmann *et al.* (2002), light conditions generally inhibit root growth, while eliminating light is beneficial for root formation. However, whole explants producing adventitious shoots were transferred to root promoting media produced roots after 12 weeks (Fig 3.28) while elongated shoots sub-cultured onto root promoting media produced roots in 7 weeks in light conditions (Fig 3.29).

4.3 Microscopy

4.3.1 The role of citric acid

The use of citric acid as an antioxidant has been shown to reduce the polymerisation of phenolics by preventing the oxidation of the phenol. However, in this case, it was unable to neutralise the phenolic compounds, and increasing concentration of the citric acid from 0.75 mg l^{-1} to 7.5 mg l^{-1}

and then to 75 mg l^{-1} did not show any improvement. Material prepared for tissue culture was dissected in a 0.75 mg l^{-1} citric acid and stored in the solution until needed. This prevented the phenolic reactions turning the cut surfaces black by reacting with the phenolics released from the ruptured cells. However, while preventing the oxidation and blackening of cut surfaces, the oxidants here did not react with the phenolics in the deeper tissues. This may be due to the citric acid being unable to penetrate the tissue, or diffuse into the organelles containing the phenolic compounds. Also, chemicals such as caffeine in combination with the fixative can help stabilise phenolic compounds in vacuoles (Kuo, 2007).

4.3.2 Glutaraldehyde fixation

Fixation with glutaraldehyde (2.5% with caffeine) and further processing of axes of *S. cordatum* using conventional TEM methods was unsuccessful (Fig 3.30), and resulted in the tissue being brittle and tearing during sectioning. The sample size was reduced to decrease the volume and to create cut surfaces for penetration of the fixative; however this was still unsuccessful. The main cause for the poor fixation was the reaction between phenolic compounds and glutaraldehyde, which formed a resin-like compound detrimental to sectioning and TEM visualisation. Additionally, the resin-like compound seemed to hinder the penetration of the fixative further into the tissue, thus leaving the deeper layers of the tissue unfixed, which contributed to poor section quality and retention of fine structure. Little detail was visible with light microscopy, and the poor fixation prevented ultra thin sections from being cut for TEM.

4.3.3 $KMnO_4$ fixation

Replacing glutaraldehyde with $KMnO_4$ did not improve fixation results (Fig 3.31). Although $KMnO_4$ is used to penetrate thick cell walls, the fixative presumably reacted with the phenolic compounds and formed a hardened compound (similar to the compound formed when aldehydes react with phenolics), that prevented ultrathin sectioning. Increasing the fixation time from 1 to 12 hours did not show a change in fixation which indicated that sufficient time was allowed for fixation. Often clarity in fixation of specimens with $KMnO_4$ is achieved through extraction of material (soluble proteins, ribosomes and RNA) (Glauert, 1980); however the phenolic compounds in specimens of *S. cordatum* were not extracted.

4.3.4 OsO_4 fixation

During fixation of material with OsO_4, penetration of the fixative was slow. The middle area of root meristematic regions of *S. cordatum* fixed with OsO_4 remained unfixed (Fig 3.32), which may indicate that sufficient time was not allowed for complete fixation. However, prolonged fixation time with OsO_4 results in polymerisation of the phenolic compounds, which could prevent the fixative penetrating further into the tissue contributing to the poor fixation of the tissue.

4.3.5 Freeze substitution

All three freeze substitution media used provided the same result. The tissue, although not showing any evidence of phenolic compounds, did show large and fine ice crystal damage (Fig 3.33a, b). Large ice crystals are created by the recrystalisation of small ice crystals formed during rapid freezing. The recrystalisation can occur during freezing or thawing of the material (Mazur, 1970), thus it is important to obtain optimum freezing and thawing rates. The addition of the high molecular weight cryoprotectant polyvinylpyrridine (PVP), did not reduce the ice crystal damage sufficiently. The freezing procedure is relatively standard, but alterations to the thawing procedure may be made. Thawing was carried out at room temperature (as samples were very small), which may have caused the small ice crystals to recrystalise and grow to form larger ice crystals, causing to damage to the tissue.

4.3.6 Heat radiation fixation

Although glutaraldehyde (2.5% with caffeine), was previously used as a fixative, the method of fixation for microwave fixation was altered. The excised meristematic region was exposed to the 2.5% glutaraldehyde for shortened periods at room temperature rather than 4^{o}C, and then microwaved for 10 s. Results were considerably improved. The effect of the phenolic compounds was not completely eradicated; however they were reduced sufficiently to permit the cutting of ultrathin sections for TEM. Presumably, the increased temperature and energy provided by the microwave increased reaction rates between the fixative and the tissue, therefore the shortened fixation period was sufficient for fixation of the tissue and not optimal for the polymerisation of the phenolic compounds. Tissue fixed at room temperature for 30 min then heat fixed (Fig 3.36) showed more phenolic deposits than tissue fixed for 5 (Fig 3.34) and 15 min (Fig 3.35) at room temperature.

CHAPTER 5: CONCLUDING COMMENTS

Seeds of *S. cordatum* have been shown to be recalcitrant in nature, as whole seeds of *S. cordatum* after being dried slowly in bags of silica gel to axis water content of 1.6 g g^{-1}, were no longer viable. However, when axes of *S. cordatum* are dried rapidly to a w.c. of 0.85 g g^{-1}, the axes showed a 10% seed viability after *in vitro* germination, which suggests that the seeds are desiccation sensitive. This also suggests that metabolism derived damage accumulates when seeds are dried slowly. However, when axes are dried rapidly, there is insufficient time for accumulation of this damage. The response to slow drying was also indicative of the accumulation of metabolism-derived damage, which is commensurate with the concept that metabolism is ongoing, additionally confirming that the seeds are recalcitrant.

The most effective method of short- to medium-term storage of seeds of *S. cordatum* is storage at original water content. The seeds remained in storage for 8 weeks without 100% germination in storage, while seeds stored in a saturated atmosphere are storable for 6 weeks before all seeds germinated in storage. The fungicide that appeared to provided best storage results was a triazole based fungicide (Orius) as this treatment showed the lowest rate of germination in storage for both storage regimes.

A long-term storage option for recalcitrant seeds is cryopreservation, however flash dried axes had lost substantial viability when dried to an axis w.c. of ≈ 2.0 g g^{-1}, which is still too high for cryopreservation (w.c. ≈ 0.3 – 0.4 g g^{-1}). An alternate option to cryostoring the zygotic axis is somatic embryos, which can be produced by manipulation of an explant using plant growth regulators (PGRs). Although an appropriate explant and correct concentrations of PGRs for somatic embryogenesis was not achieved and hence material suitable for cryopreservation was not produced; however, a protocol for organogenesis was developed. Excised embryonic axes can be induced to produce microshoots when cultured onto media containing full strength MS, 1 mg.l^{-1} BAP and 0.5 mg.l^{-1} NAA. These microshoots elongate when sub-cultured onto the same medium or PGR free medium with ½ or full strength MS. Excised elongated shoots can produce roots when sub-cultured onto media containing either full strength MS, 0.5 mg l^{-1} GA_3 and 30 g l^{-1} sucrose or full strength MS, 0.2 mg l^{-1} GA_3 and 30 g l^{-1} sucrose, and whole explants of multiple shoots can

develop roots when sub-cultured onto media containing full strength MS, 0.1 mg l^{-1} and 30 g l^{-1} sucrose.

The additional aim of this project was to develop a suitable method for TEM fixation as it became evident that a sound understanding of the ultrastructural nature of the tissue was necessary. Conventional methods of fixation with the primary fixative glutaraldehyde, and post-fixing with OsO_4, were unsatisfactory. The high phenolic content of the cells did not allow for adequate fixation which resulted in poor infiltration of all processing solvents. Other primary fixatives including $KMnO_4$ and OsO_4 were tested and eventually, a new method of fixation, cryo-fixation followed by freeze substitution, was tested. However results were unsatisfactory due to ice damage and microwave fixation was attempted. Adequate results were produced after 15 min fixation followed by microwaving for 10 s, and ultra-thin sections for TEM viewing were obtainable.

To improve TEM processing results, an industrial microwave with variable power settings, circulating water and a cold plate below samples to prevent hot spots (which can be detrimental to samples), should be used. Additionally, a vacuum can be applied to enhance infiltration of samples. Further investigations into cryo-fixation methods, using high pressure freezing and a slow increase of temperature, over several days during infiltration of material with fixatives and dehydrating solutions, could be tried.

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